

Description

EXPRESSION OF BIOLOGICALLY ACTIVE PDGF ANALOGS IN EUKARYOTIC CELLS

Technical Field

The present invention relates to the production of PDGF analogs in general, and more specifically, to the expression of biologically active PDGF analogs in eucaryotes.

Background Art

Human platelet derived growth factor (PDGF) has been shown to be the major mitogenic protein in serum for mesenchymal derived cells. This is well documented by numerous studies of platelet extracts or purified PDGF induction of either cell multiplication or DNA synthesis (a pre-requisite for cell division) in cultured smooth muscle cells, fibroblasts and glial cells (Ross et al., PNAS 71: 1207, 1974; Kohler and Lipton, Exp. Cell Res. 87: 297, 1974; Westermarck and Wasteson, Exp. Cell Res. 98: 170, 1976; Heldin et al., J. Cell Physiol. 105: 235, 1980; Raines and Ross, J. Biol. Chem 257: 5154, 1982). Furthermore, PDGF is a potent chemoattractant for cells that are responsive to it as a mitogen (Grotendorst et al., J. Cell Physiol. 113: 261, 1982; Seppa et al., J. Cell Biol. 92: 584, 1982). It is not generally the case that mitogens also act as chemotactic agents. Due to its mitogenic activity, PDGF is useful as an important component of a defined medium for the growth of mammalian cells in culture, making it a valuable research reagent with multiple applications in the study of animal cell biology.

In vivo, PDGF normally circulates stored in the alpha granules of platelets. Injury to arterial endothelial linings causes platelets to adhere to the exposed connec-

tive tissue and release their granules. The released PDGF is thought to chemotactically attract fibroblasts and smooth muscle cells to the site of injury and to induce their focal proliferation as part of the process of wound repair (Ross and Glomset, N. England Journal of Medicine 295: 369, 1976).

It has been postulated that as a part of this response to injury, PDGF released by platelets may play a causative role in the development of the proliferative lesions of atherosclerosis (Ross and Glomset, *ibid.*) which is one of the principal causes of myocardial and cerebral infarction. Strategies for the prophylaxis and treatment of atherogenesis in the past have been narrowly directed toward reducing risk factors for the disease, such as lowering blood pressure in hypertensive subjects and reducing elevated cholesterol levels in hypercholesterolemic subjects.

Recent studies have shown that one of the two protein chains comprising PDGF and the putative transforming protein of simian sarcoma virus (SSV), an acute transforming retrovirus, appear to have arisen from the same or closely related cellular genes. In particular, computer analysis of a partial amino acid sequence of PDGF has revealed extensive homology with the gene product, p28^{sis}, of SSV (Doolittle, Waterfield and Johnsson, *ibid.*). Further, more recent studies have illustrated that p28^{sis} and PDGF show antigenic as well as structural similarities (Robbins et al., Nature 305: 605, 1983; Niman, Nature 307: 180, 1984).

Although previous attempts, such as that summarized in Devare et al., (Cell 36: 43, 1984), have been made to express the v-sis gene in a transformed microorganism, they have not been successful in producing mitogenic material. More recently, investigators have described the production of p28^{sis} in E. coli as a fusion protein. (Wang et al., J. Biol. Chem 259: 10645, 1984).

This protein appears to compete with PDGF for binding to PDGF receptor sites. While SSV transformed rodent cells have been shown to exhibit a mitogenic activity similar to PDGF (Deuel et al., Science 221: 1348, 1983; Owen et al., Science 225: 54, 1984), it is not clear that this activity is due to a gene product from SSV (i.e., p28^{sis}). Furthermore, cells transformed by a variety of viruses other than SSV produce a PDGF-like mitogen into the culture medium (Bowen-Pope et al., PNAS 81: 2396, 1984).

While natural PDGF may be isolated from human plasma or platelets as starting material, it is a complex and expensive process, in part due to the limited availability of the starting material. In addition, it is difficult to purify PDGF with high yield from other serum components due to its extremely low abundance and biochemical properties. Furthermore, the therapeutic use of products derived from human blood carries the risk of disease transmission due to contamination by, for example, hepatitis virus, cytomegalovirus, or the causative agent of Acquired Immune Deficiency Syndrome (AIDS).

In view of PDGF's clinical applicability in the treatment of injuries in which healing requires the proliferation of fibroblasts or smooth muscle cells and its value as an important component of a defined medium for the growth of mammalian cells in culture, the production of useful quantities of protein molecules similar to authentic PDGF which possess mitogenic activity is clearly invaluable.

In addition, the ability to produce relatively large amounts of PDGF would be a useful tool for elucidating the putative role of the v-sis protein, p28^{sis}, in the neoplastic process.

Further, since local accumulation of smooth muscle cells in the intimal layer of an arterial wall is central to the development of atherosclerotic lesions (Ross and Glomset, *ibid.*), one strategy for the prophylaxis and treatment of atherosclerosis would be to suppress smooth

muscle cell proliferation. The ability to produce large amounts of PDGF would be useful in developing inhibitors or designing specific approaches which prevent or interfere with the in vivo activity of PDGF in individuals with atherosclerosis.

Disclosure of The Invention

Briefly stated, the present invention discloses a DNA construct capable of directing the expression and secretion of biologically active PDGF analogs in eucaryotic cells. The DNA construct contains a transcriptional promoter followed downstream by a gene encoding a protein having substantially the same structure and/or mitogenic activity as PDGF, and a signal sequence capable of directing the secretion of the protein from the eucaryotic cell. The gene may be the v-sis gene or a derivative of the v-sis gene of simian sarcoma virus or portions thereof which encode a protein having biological activity. Further, the derivative of the v-sis gene may be the portion of the v-sis gene which is substantially homologous to the B chain of PDGF. In addition, the gene may be the human cDNA gene for PDGF or portions thereof encoding a protein having biological activity.

Another aspect of the invention discloses a method of preparing biologically active PDGF analogs by introducing into a eucaryotic host a DNA construct capable of directing the expression and secretion of biologically active PDGF analogs in eucaryotic cells. The DNA construct contains a transcriptional promoter followed downstream by a gene encoding a protein having substantially the same structure and/or mitogenic activity as PDGF and a signal sequence capable of directing the secretion of the protein from the eucaryotic cell. Subsequent to introducing the DNA construct into the eucaryotic host, the method includes growing the eucaryotic host in an appropriate medium and then isolating the protein product of the gene from the

eucaryotic host. Eucaryotic host cells transformed with such a DNA construct are also disclosed.

5 The present invention further provides a method for promoting the growth of mammalian cells through incubating the cells with a biologically active PDGF analog expressed by a eucaryotic cell transformed with a DNA construct capable of directing the expression and secretion of biologically active PDGF analogs in eucaryotic cells. 10 The DNA construct contains a transcriptional promoter followed downstream by a gene encoding a protein having substantially the same structure and/or mitogenic activity as PDGF and a signal sequence capable of directing the secretion of the protein from the eucaryotic cell.

15 In one embodiment of the invention, the eucaryotic cell may be a yeast cell, and the DNA construct more appropriately termed an extrachromosomal element.

Other aspects of the invention will become evident upon reference to the following detailed description and attached drawings. 20

Brief Description of The Drawings

Figure 1A is a schematic restriction map of the proviral genome of SSV.

25 Figure 1B depicts the nucleotide sequence and predicted amino acid sequence encoded by the v-sis region of the SSV genome.

Figure 2 illustrates the construction of a plasmid which contains the MF01 promoter and secretory signal sequence upstream of the v-sis gene. 30

Figure 3 illustrates the construction of plasmid p192.

Figure 4 illustrates the oligonucleotide directed deletion mutagenesis of the amino terminal sixty-six v-sis codons. 35

Figure 5 illustrates the construction of plasmid p270.

Figure 6 illustrates the insertion of v-sis expression units upstream of the TPI terminator.

Figure 7 illustrates the replacement of the M Φ 1 promoter with the TPI promoter and inclusion of the VS2 α construction in the pCPOT vector.

Figure 8 illustrates the construction of plasmid pTVS2 α T.

Figure 9 illustrates the construction of a B chain expression unit VSB and its introduction into the pMPOT2 vector.

Figure 10 depicts the electrophoretic and subsequent hybridization analysis of total RNA isolated from a yeast host transformed with various plasmids probed with a nick-translated v-sis gene fragment.

Figure 11 depicts the results of an ELISA of concentrated culture media from the yeast transformants containing plasmids pVS α , pVS2 α , pl17-2 and pCPOT.

Figure 12 is a dose response curve of mitogenic activity of concentrated culture media from yeast transformants containing plasmids pVS α and pl17-2, compared to purified PDGF.

Figure 13 is a dose response curve of PDGF receptor binding by media concentrates from yeast transformants containing plasmids pVS α m, pVS2 α m, pVSBm and pMPOT2 compared to authentic PDGF.

Figure 14 is a dose response curve of mitogenic activity of media concentrates from yeast transformants containing plasmids pVS α m, pVS2 α m, pVSBm, and pMPOT2 compared to authentic PDGF.

Figure 15 is a graph illustrating the mitogenic activity of the VSB encoded protein fractionated by polyacrylamide gel electrophoresis.

Best Mode For Carrying Out the Invention

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms to be used hereinafter.

Polypeptide: A polymer of amino acids.

Reading Frame: The arrangement of nucleotide codons which encode an uninterrupted stretch of amino acids. During translation of a mRNA, the proper reading frame must be maintained. For example, the sequence GCUGGUUGUAAG may be translated into three reading frames or phases, depending on whether one starts with G, with C, or with U, and thus may yield three different peptide products. Translation of the template begins with an AUG codon, continues with codons for specific amino acids, and terminates with one of the translation termination codons.

Coding Sequence: DNA sequences which in the appropriate reading frame directly code for the amino acids of a protein.

Complementary DNA: or cDNA. A DNA molecule or sequence which has been enzymatically synthesized from the sequences present in a mRNA template.

Secretory Signal Sequence: That portion of a gene encoding a signal peptide. A signal peptide is the amino acid sequence in a secretory protein which signals its translocation into the secretory pathway of the cell. Signal peptides generally occur at the beginning (amino terminus) of the protein and are 20-40 amino acids long with a stretch of 9-10 hydrophobic amino acids in their center. Very often the signal peptide is proteolytically cleaved from the protein during the process of secretion.

Cell Surface Receptor: A protein molecule at the surface of a cell which specifically interacts with or binds a molecule approaching the cell's surface. Once the
5 receptor has bound the cognate molecule, it effects specific changes in the physiology of the cell.

Mitogen: A molecule which stimulates cells to undergo mitosis. Mitosis is asexual somatic cell division
10 leading to two daughter cells, each having the same number of chromosomes as the parent cell.

Transformation: The process of stably and hereditably altering the genotype of a recipient cell or
15 microorganism by the introduction of purified DNA. This is typically detected by a change in the phenotype of the recipient organism.

Transcription: The process of producing a mRNA
20 template from a structural gene.

Expression: The process, starting with a structural gene, of producing its polypeptide, being a combination of transcription and translation. An expression
25 vector is a plasmid derived construction designed to enable the expression of a gene carried on the vector.

Plasmid: An extrachromosomal double stranded DNA sequence comprising an intact "replicon" such that the
30 plasmid is replicated in a host cell. When the plasmid is placed within a unicellular organism, the characteristics of that organism may be changed or transformed as a result of the expression of the DNA sequences of the plasmid. For example, a plasmid carrying the gene for tetracycline
35 resistance (tet^R) transforms a cell previously sensitive to tetracycline into one which is resistant to it.

Yeast Promoter: DNA sequences upstream from a yeast gene which promote its transcription.

5 Biological Activity: Some function or set of activities performed by a molecule in a biological context (i.e., in an organism or an in vitro facsimile). In the case of PDGF, these biological activities include binding to cell surface receptor molecules, inducing chemotaxis and
10 inducing mitogenesis of responsive cell types.

As noted above, human platelet derived growth factor (PDGF) has been shown to be a major mitogenic protein in serum. PDGF is known to be composed of two
15 polypeptide chains, an A chain and a B chain, which are held together by disulfide bonds to form the biologically active molecule. The A chain and B chain alone do not appear to exhibit any mitogenic activity, (Raines and Ross, *ibid.*) and attempts to reconstitute activity by reoxidation
20 of the reduced polypeptides have not been successful. Recently, the amino acid sequence of the B chain has been shown to be substantially homologous to a portion of the v-sis gene product, p28^{sis} (Doolittle et al., Science 221: 275, 1983; Waterfield et al., Nature 304: 35, 1984; and
25 Johnsson et al., Embo 3: 921, 1984). The homology between these two proteins strongly suggests that they are derived from the same or closely related cellular genes.

Given the fact that biologically active PDGF was known to contain equimolar amounts of A and B chain, and
30 that previous attempts directed toward expressing v-sis sequences in E. coli did not yield mitogenic material, it would not be expected that merely expressing a portion of the v-sis gene homologous to a portion of the PDGF gene in a microorganism would result in a molecule which exhibited
35 mitogenic activity. The present invention however, unlike the previous attempts noted above, was designed to express the v-sis gene or portions thereof absent of heterologous

sequences, such that the expressed molecules are more closely related to the B chain of PDGF. Further, the expression system of the present invention was designed to
5 produce the gene product via a eucaryotic secretory pathway. This enables the expressed protein molecules to be properly processed and assembled such that they exhibit biological activity. Indeed, the present invention, in contrast to
10 previous efforts, results in the secretion of PDGF analogs which are biologically active.

In its active form, PDGF is a heat stable protein composed of heterogeneously sized species of between 28,000 and 31,000 Daltons, all of the individual species being active in stimulating DNA synthesis (Raines and Ross,
15 *ibid.*; Deuel et al., *J. Biol. Chem* 256: 8896, 1981; Antoniadou, *PNAS* 78: 7314, 1981). Where individual species with molecular weights of 27,000; 28,500; 29,000; and 31,000 Daltons have been isolated and assayed, they have been found to have comparable mitogenic activity and amino acid
20 composition (Raines and Ross, *ibid.*) Further, these species show extensive tryptic peptide homology. The slight variations in size among the species are most probably due to differences in carbohydrate composition and proteolysis.

25 Through studies of PDGF which has been extensively purified from platelet-rich human plasma, it is likely, as noted above, that PDGF is composed of two polypeptide chains, an A chain (14,000 Daltons) and a B chain (16,000 Daltons), which are disulfide bonded together to form the
30 biologically active dimer molecule (Raines & Ross, Deuel et al., Antoniadou, *ibid.*). The PDGF nomenclature found in the literature is not consistent (Doolittle et al., Waterfield et al., Raines and Ross, Johnsson et al., *ibid.*). The nomenclature of Johnsson et al. (*ibid.*) has been
35 adopted, wherein the two polypeptides found in pure PDGF are called "A chain" and "B chain." The B chain is homologous to p28^{sis} and was previously called "peptide I"

(Waterfield et al., *ibid.*) or "1a" (Doolittle et al., *ibid.*). The A chain was previously termed "peptide II" (Waterfield et al., *ibid.*) or "2a" (Doolittle et al., *ibid.*).
5 Data derived from a partial amino acid sequence of PDGF indicate that the two polypeptide chains (A chain and B chain) show some homology (Doolittle et al., *ibid.*, Waterfield et al., *ibid.*, and Johnsson et al., *ibid.*, Antoniades and Hunkapiller, Science **220**: 963, 1983). The A
10 chain and B chain alone do not appear to exhibit any mitogenic activity, and attempts to reconstitute activity by reoxidation of the reduced polypeptides have not been successful (Raines & Ross, *ibid.*).

The v-sis gene, as mentioned above, is the
15 transforming gene of simian sarcoma virus (SSV). The v-sis gene has been cloned and its DNA sequence determined (Devare et al., PNAS **79**: 3179, 1982; Devare et al., PNAS **80**: 731, 1983). Analysis of this sequence revealed an open reading frame which could encode a 28,000 Dalton protein,
20 designated p28^{sis}. Subsequently, such a protein was identified in SSV infected cells (Niman, *ibid.*; Robbins, *ibid.*). The predicted amino acid sequence of the v-sis gene product, p28^{sis}, was found to have a high degree of homology with the actual amino acid sequence of a portion of the B
25 chain of PDGF (Johnsson, *ibid.*). The homology of the PDGF B chain to the v-sis gene product begins at amino acid 67 of p28^{sis}, a serine, and continues for approximately 109 amino acids to a threonine residue at amino acid 175. The amino acid sequences preceding and following the B chain
30 homologous region of p28^{sis} are not homologous to either the A or B chains of mature PDGF (Johnsson, *ibid.*) In addition, PDGF and p28^{sis} have been shown to be similar antigenically (Niman, *ibid.*; Robbins, *ibid.*). The v-sis gene product, p28^{sis}, a protein of approximately 225 amino
35 acids, appears to be proteolytically processed to a protein of approximately 20,000 Daltons (p20^{sis}) in SSV infected cells (Niman, *ibid.*; Robbins, *ibid.*). This 20,000 Dalton

protein can be immunoprecipitated with antiserum against PDGF.

As noted above, previous attempts at expressing
5 v-sis sequences in prokaryotes did not yield biologically active material. Further, the v-sis gene product p28^{Sis}, as well as PDGF itself, are secreted mammalian proteins. In order to achieve biologically active material, the present invention utilizes the secretory pathway of
10 eucaryotic cells to express the v-sis gene and derivatives of the v-sis gene. Expression and secretion of the v-sis gene product from a eucaryotic cell enables processing and assembly which results in molecules with native and active conformation.

15 The secretory pathways of most eucaryotes are believed to be similar. In particular, mammalian cell and yeast cell secretory pathways are well characterized and are homologous. The presence of a secretory signal sequence on the expressed polypeptide is an important
20 element in eucaryotes, due to its role in introducing the molecule into the secretory pathway, thereby leading to proper assembling and processing. Provided that appropriate transcriptional promoter and secretory signal sequences are utilized, generally any eucaryote could express and
25 secrete the v-sis gene product in a biologically active form.

An easily manipulable and well characterized eucaryote is the yeast cell. For these reasons, yeast was chosen as a model example of an appropriate eucaryotic cell
30 within the present invention. Accordingly, the v-sis gene and fragments thereof encoding the 109 amino acids with homology to the PDGF B chain were inserted into yeast extrachromosomal elements containing a yeast promoter capable of directing the expression of biologically active
35 PDGF analogs. In accordance with the present invention, the yeast promoter is followed downstream by a fragment of

the v-sis gene which encodes a protein having substantially the same structure and/or mitogenic activity as PDGF.

5 Genes which encode a protein having substantially the same structure and/or mitogenic activity as PDGF include the v-sis gene or a derivative of the v-sis gene of simian sarcoma virus (SSV) or portions thereof or the human cDNA gene for PDGF or portions thereof. Specifically, DNA sequences encoding polypeptides substantially homologous to
10 the B chain of PDGF are preferred. The genes to be utilized in the extrachromosomal element may be isolated using standard recombinant DNA techniques.

The human PDGF cDNA gene may be isolated from a human cDNA library made from an appropriate source of messenger RNA by using the v-sis gene or a fragment thereof as
15 a hybridization probe. A preferred source of mRNA is human umbilical vein endothelial cells. These cells can be cultured in vitro for short periods of time and are known to secrete PDGF into the culture medium (DiCorleto and Bowen-
20 Pope, PNAS 80: 1919, 1983). The identity of this cDNA gene as that encoding PDGF may be verified by DNA sequencing.

Promoters which may be utilized in yeast include the yeast alpha-factor (MF α 1) promoter and the yeast triose phosphate isomerase (TPI) promoter. Promoters may also be
25 obtained from other yeast genes, e.g., Alcohol Dehydrogenase 1 (ADH1), Alcohol Dehydrogenase 2 (ADH2).

The constructions described herein were designed such that the v-sis gene product would be secreted from the yeast cell into the media. This was accomplished through
30 use of the secretory signal sequence of the yeast mating pheromone alpha-factor (Kurjan and Herskowitz, Cell 30: 933, 1982; Julius et al., Cell 36: 309, 1984; and Brake et al., PNAS 81: 4642, 1984) although other secretion signals may be used. To ensure the efficient transcription termination and polyadenylation of mRNA, a yeast terminator
35 sequence, such as the triose phosphate isomerase termi-

nator, was added. (Alber and Kawasaki, J. Molec. Genet. Appl. 1: 419, 1982.)

Once an appropriate DNA fragment containing the
5 gene of interest is identified, it is ligated to an appropriate promoter and secretory signal sequence. Methods of ligation of DNA fragments have been amply described (Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory 1982) and are well within the
10 skill of those of ordinary skill in the art to perform. After preparation of the v-sis expression constructions, the constructs are inserted into a yeast expression vector.

The replicating plasmid YEpl3, containing an origin of replication and a selectable marker, the LEU2
15 gene, was used for the initial expression constructions. The use of the selectable marker LEU2 in yeast cells deficient in their ability to synthesize leucine allows for the positive selection of those cells containing the LEU2 plasmid by their ability to grow on minus leucine growth
20 media. Although these constructions directed the expression of a product having some mitogenic activity, it is preferable to use an expression vector which is more stably maintained within the host cell in order to produce more mitogenic activity per culture.

25 Suitable yeast expression vectors in this regard are the plasmids pCPOT and pMPOT2, which include the Schizosaccharomyces pombe gene encoding the glycolytic enzyme triose phosphate isomerase (POT1 gene). Inclusion of the POT1 gene ensures the stable maintenance of the plasmid in
30 an appropriate host cell due to its ability to complement the corresponding gene deletion present within this host cell. In addition, the MFal promoter was replaced by the Saccharomyces cerevisiae TPI promoter with the intention of further increasing transcription and expression.

35 After preparation of the DNA construct incorporating the TPI promoter, the alpha factor secretory signal sequence, the appropriate segment of the v-sis gene or the

human cDNA gene for PDGF, and the TPI terminator in an appropriate vector, the construct is transformed into the yeast host with a TPI deletion. Procedures for transforming yeast are well known in the literature.

The transformed yeast cells may be selected by growth on conventional complex medium containing glucose when the pCPOT vector is utilized. A conventional medium such as YEPD (20 grams glucose, 20 grams Bacto-peptone, 10 grams yeast extract per liter) may be used. Once selected, transformants containing the v-sis expression constructions are grown to stationary phase on conventional complex media, the cells removed, and the medium concentrated. Noting that authentic human PDGF is a highly cationic and hydrophobic protein (Raines and Ross, *ibid.*; Antoniades, *ibid.*; Deuel et al., 1981, *ibid.*), it was expected that the putative yeast product would possess similar characteristics, allowing it to be concentrated on a hydrophobic chromatography matrix such as C8-Sepharose (Pharmacia Fine Chemicals AB, Uppsala, Sweden).

Using a variety of assays, it is demonstrated that growth media from yeast cultures expressing the v-sis derivatives possess biological activities identical to authentic human PDGF.

Expression of biologically active v-sis derivatives in eucaryotic cells other than yeast can be achieved by a person skilled in the art by using the appropriate expression/regulatory signals. Transcriptional promoters capable of directing the expression of v-sis sequences are chosen for their ability to give efficient and/or regulated expression in the particular eucaryotic cell type. Signal sequences capable of directing the v-sis gene product into the cell's secretory pathway are chosen for their function in the appropriate cell type. Other useful regulatory signals, such as transcription termination signals, polyadenylation signals and transcriptional enhancer sequences, are also chosen for their function in the appropriate cell

type, the selection of which would be apparent to an individual skilled in the art.

The techniques of cell culture have advanced considerably in the last several years as have the number and varieties of mammalian cells which will grow in culture. Central to these advances is a better understanding of the nutritional requirements (i.e., hormones and growth factors) of cultured cells (Barnes and Sato, Cell 22: 649, 1980). The types of cells able to grow in culture can be crudely classified in two groups: normal and transformed. So-called "normal" cells are generally not immortal in culture, they do not form tumors when injected into animals and they retain a normal diploid karyotype. Normal cells may also retain much of their differentiated character in culture. Within the category of normal cells are those which will only grow for a limited number of generations in culture, termed "cell strains" or "primary cultures." Some normal cell lines, while not meeting all the criteria of transformation, may grow indefinitely in culture. Transformed cells are immortalized for growth in culture, typically have lost their differentiated phenotype, and have acquired karyotypic aberrations. They may also be independent of anchorage for growth and induce tumors when injected into the appropriate host animal. Cells in any of these categories which grow in vitro and possess PDGF receptors will be responsive to the PDGF analogs of this invention in culture.

To summarize the examples which follow, EXAMPLE I demonstrates the construction of a v-sis subclone of pSSV-11 in the E. coli replicating plasmid pUC13, subsequently designated pVSIS/Pst. EXAMPLE II demonstrates the construction of the plasmid pVSa, which includes the ligation of v-sis to the Mfal promoter and secretory signal sequence. EXAMPLE III demonstrates the oligonucleotide directed deletion mutagenesis of the first 195 base pairs of the v-sis gene using a technique which employs single

stranded bacteriophage M13, in order to eliminate the first sixty-six amino acids of the v-sis gene product, p28^{sis}, which are not homologous to the B chain of PDGF. A resulting phage with the correct deletion was designated m11vs2 α . EXAMPLE IV demonstrates the incorporation of the v-sis related constructions described in Examples II and III into the yeast replicating vector YEpl3 and addition of yeast TPI terminator sequences. Subsequently, VS2 α sequences were inserted into the plasmid pCPOT, which ensures the stable maintenance of the plasmid in the host cell. This plasmid was designated pll7-2. This example also demonstrates the construction of the plasmid pVSB and the expression vector pMPOT2. EXAMPLE V demonstrates the transformation of yeast host cells with the plasmids YEpVS α , YEpVS2 α , pll7-2 and control plasmids p270 and pCPOT, and subsequent transcriptional analysis. EXAMPLE VI demonstrates the concentration of the spent yeast growth media from cultures containing the v-sis expressing transformants and their subsequent analysis for PDGF-like material by the ELISA, radioreceptor and mitogenesis assays. Clear evidence is presented that these yeast media containing the v-sis related gene products described herein possess biological activities identical to authentic human PDGF.

The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLES

Unless otherwise indicated, standard molecular biological methods were used. Restriction endonucleases and other DNA modification enzymes (i.e., T₄ polynucleotide kinase, calf alkaline phosphatase, Klenow DNA polymerase) were obtained from Bethesda Research Laboratories, New England Biolabs, Boehringer-Mannheim or Collaborative Research and were used as the manufacturer suggested unless indicated otherwise. M13 phage and pUC plasmid vectors and

appropriate host strains were obtained from Bethesda Research Laboratories. E. coli cultures were transformed by the calcium chloride method of Dagert and Ehrlich (Gene 5 6: 23, 1979). Yeast cultures were transformed as described by Beggs (Nature 275: 104, 1978). Plasmid and M13 replicative form (RF) DNA were prepared from E. coli transformants by the method of Birnboim and Doly (Nucleic Acids Research 7: 1513, 1979). Single stranded M13 phage DNA was prepared as described by S. Anderson (Nucleic Acids Research 10 13: 3015, 1981). DNA fragments were extracted from agarose gels by the method of J. Langridge et al. (Analyt. Biochem. 103: 264, 1980). DNA sequencing was performed by the dideoxy method on M13 templates (Messing, Meth. in Enzymology 15 101: 20, 1983).

EXAMPLE I

Subcloning of V-SIS from pSSV-11

20

The SSV retroviral genome was cloned from SSV-11 nonproductively infected normal rat kidney (NRK) cells which had SSV integrated into their genome (Devare et al., 1982, *ibid.*). The SSV DNA was isolated as a 5.8 kilobase 25 (kb) Eco RI fragment and subsequently inserted into the plasmid pBR322, resulting in the clone pSSV-11. This clone was obtained from S. Aaronson (National Institutes of Health, Bethesda, MD).

Figure 1A is a schematic restriction map of the 30 5.8 kilobase proviral genome of SSV. Only the restriction sites relevant to the present invention are indicated. The open box designates the p28^{sis} coding portion of the v-sis gene.

Figure 1B depicts the nucleotide sequence of the 35 v-sis gene and some flanking SSV sequences. The v-sis gene is inserted 19 nucleotides 3' of the putative ATG initiation codon of the envelope (env) gene of SSV (Devare et

al., 1982, *ibid.*). It is believed that transcription and translation of v-sis sequences are directed by SSV sequences resulting in an env-sis fusion protein. The
5 nucleotide sequence shown in Figure 1B is corrected from that published by Devare et al. in 1982 (*ibid.*). The corrections include those made by Devare et al. in 1983 (*ibid.*) and by the inventors herein. The original numbering scheme of Devare et al. (1982, *ibid.*) is retained here
10 for ease of reference. The numbers assigned to the restriction sites in Figure 1A are from Figure 1B.

A subclone of pSSV-11 (Figure 2) containing a portion of the v-sis gene was constructed in the E. coli replicating plasmid pUC13 (Vieira and Messing, Gene, 19:
15 259, 1982; and Messing, Meth. in Enzymology 101: 20, 1983). Five micrograms (ug) of pSSV-11 was digested with the restriction endonuclease Pst I and the 1.2 kb fragment containing sequences numbered 454-1679 (Figure 1) was purified by agarose gel electrophoresis (0.9%) and extracted from
20 the gel with cetyltrimethylammonium bromide (CTAB) plus butanol (Langridge et al., *ibid.*). Two ug of pUC13 was also digested with Pst I, phenol/chloroform (CHCl₃) extracted and ethanol (EtOH) precipitated. Forty ng of the 1.2 kb v-sis fragment and 50 ng of Pst I cut pUC13
25 were ligated overnight at room temperature with 40 units (u) of T₄ DNA ligase. The ligation mixture was used to transform E. coli K-12 strain JM83 (Messing, Recombinant DNA Technical Bulletin, NIH Publication No. 79-009, 2, No. 2, 43-48, 1979) in the presence of 5-bromo,4-chloro, 3-
30 indolyl- β -D-galactoside (X-gal) and isopropyl β -D-thiogalactoside (IPTG). Plasmid DNA prepared from ampicillin resistant white colonies was digested with Pst I to verify the presence of the insert and the resulting plasmid was designated pVSIS/Pst.

EXAMPLE II

Construction of the Plasmid pVSo

5

A. Preparation of V-SIS for Fusion to MFol.

Six hundred ug of plasmid pSSV-11 (Figure 2) was digested with restriction endonucleases Bam HI and Pvu II in 200 microliters (ul) of 50 mM NaCl, 10 mM MgCl₂, 10 mM Tris pH 7.5 (medium salt buffer), and 100 ug/ml bovine serum albumin (BSA), overnight at 37°C. The digestion products were electrophoresed through a 1.1% agarose gel and the 1100 base pair (bp) Bam HI-Pvu II fragment (Figure 2) cut out, extracted and EtOH precipitated. The DNA pellet was dissolved in 75 ul Hph I buffer to which was added 20 ul of 1 mg/ml BSA and 5 ul Hph I. After overnight digestion at 37°C the mixture was electrophoresed through a 1.25% agarose gel and the 396 bp Hph I-Pvu II fragment isolated from the gel and EtOH precipitated. The DNA pellet was dissolved in 30 ul of Klenow buffer (6mM Tris pH 7.5, 6 mM MgCl₂, 60 mM NaCl) and the 3' overhanging nucleotide at the Hph I cleavage site removed by treatment with 5 u of Klenow polymerase for 5 minutes at 37°C. One ul of a mixture containing all four deoxyribonucleotides each at 1 mM was added and the reaction mixture incubated an additional 10 minutes. After phenol/CHCl₃/ether (Et₂O) extraction and EtOH precipitation, the DNA pellet was dissolved in 30 ul of medium salt buffer and digested with 5 u of Bgl II for three hours at 37°C. The DNA was electrophoresed through a 1.25% agarose gel and the 269 bp Hph I - Bgl II fragment extracted and EtOH precipitated. The Hph I cleavage terminus of this Klenow blunted fragment begins with the tri-nucleotide sequence

35

5'ATG.....(Figure 2)

3'TAC.....

B. MFal Promoter and Secretory Leader Fragment.

Plasmid p192 (Figure 3) comprises a portion of
5 the gene for the yeast mating pheromone α -factor (MFal
gene) cloned in the bacterial plasmid pUC13 (Vieira and
Messing, *ibid.*; and Messing, Meth. in Enzymology 101: 20,
1983). Cloning of the MFal gene from a genomic library has
been described by Kurjan and Herskowitz (*ibid.*). The gene
10 was isolated in this laboratory in a similar manner, using
as starting material a yeast genomic library of partial *Sau*
3A fragments cloned into the *Bam* HI site of YEpl3 (Nasmyth
and Tatchell, Cell 19: 753, 1980). From this library, a
plasmid was isolated which expressed α -factor in a diploid
15 strain of yeast homozygous for the mat α 2-34 mutation
(Manney et al., J. Cell Biol 96: 1592, 1983). The clone
contained an insert overlapping with the MFal gene charac-
terized by Kurjan and Herskowitz (*ibid.*). This plasmid,
known as pZA2 (Figure 3), was cut with *Eco* RI and the 1700
20 bp fragment comprising the MFal gene was purified. This
fragment was then subcloned into the *Eco* RI site of pUC13
to produce the plasmid p192.

Fifteen μ g of plasmid p192 was digested in 30 μ l
of medium salt buffer with 20 units of *Hind* III overnight
25 at 37°C. The reaction mixture was diluted to 60 μ l with
Klenow buffer and the four deoxyribonucleotides added to a
final concentration of 50 μ M each. Ten units of *Klenow*
polymerase were added to the ice-cold mixture and incuba-
tion allowed to proceed 12 minutes at 15°C. Following
30 phenol/*CHCl*₃/*Et*₂O extraction, the aqueous phase was
concentrated by lyophilization to a volume of 10 μ l and
digested with 20 units of *Eco* RI for 70 minutes at 37°C.
The products were electrophoresed through a 0.9% agarose
gel and the 1.2 kb *Eco* RI-*Hind* III (blunted) MFal fragment
35 extracted and *EtOH* precipitated. This DNA fragment contains
the transcriptional promoter and secretory signal sequences
of MFal.

C. Preparation of v-sis 3' Sequences and Cloning
Vector pUC12; Fragment Ligation.

5

Twenty ug of plasmid pVSIS/Pst was digested with Bgl II and Xba I in 40 ul of medium salt buffer. Subsequent electrophoresis through 1% agarose, extraction of the DNA and EtOH precipitation provided the purified v-sis 756 bp Bgl II-Xba I fragment (Figure 2). E. coli replicating plasmid pUC12 (5 ug) was digested with Eco RI and Xba I and gel purified as above (Figure 2).

Referring to Figure 2, equimolar amounts of the four DNA fragments described above, adjusted to 10 ng of the 296 bp Hph I-Bgl II v-sis fragment, were mixed in 15 ul of ligase buffer (6 mM Tris pH 7.6, 6.6mM MgCl₂, 0.4 mM ATP, 2 mM spermidine, 20 mM DTT, and 100 ug/ml BSA) and ligated with 40 units of T₄ DNA ligase overnight at 14°C. The reaction mixture was brought to room temperature, an additional 150 units of T₄ ligase added, and incubated 10 more hours. Seven ul of the ligation mix was used to transform E. coli K-12 RR1 (ATCC #31343; Bolivar, E. et al., Gene 2: 95, 1977), and ampicillin resistant transformants selected. Plasmid DNA was prepared from 12 such bacterial colonies and digested with Xba I. Two clones gave a ~2.2 kb band predicted by the proper fragment alignment (Figure 2). Further analysis of these by Bgl II-Xba I restriction mapping gave expected bands of approximately 1.5 kb from the MFal/v-sis fusion and 760bp for the Bgl II-Xba I v-sis fragment. DNA sequence analysis verified the desired nucleotide sequence at the MFal/v-sis junction. The resultant plasmid was designated pVS α .

35

EXAMPLE III

5 Oligonucleotide Directed Deletion Mutagenesis of
 66 Amino Terminal v-sis Codons

 Homology between the v-sis protein p28^{sis} and
PDGF begins at amino acid 67 of p28^{sis}, a serine residue
corresponding to the NH₂ terminal residue of the PDGF B
10 chain (Johnsson, *ibid.*)

 Proteolytic processing of the MFol primary trans-
lation product occurs at the Lys-Arg cleavage signal 85
amino acids from the initiator methionine (Kurjan and
Herskowitz, *ibid.*). A v-sis derivative was constructed in
15 which the first 66 codons of p28^{sis} were removed such that
serine residue 67 of v-sis immediately follows the MFol
Lys-Arg processing signal.

 Referring to Figure 4, approximately 40 ng of the
gel purified 2.2 kb Xba I fragment of pVS α was ligated with
20 120 ng of Xba I digested, alkaline phosphatase treated
M13mpl1 DNA (Messing, Meth. in Enzymology, *ibid.*). The
ligation mixture was used to transform E. coli K-12 strain
JM101 (ATCC 33876) in the presence of X-gal and IPTG.
Isolated white plaques were picked and used to infect 3 ml
25 cultures of log phase growth JM101 cells. Replicative form
(RF) DNA was prepared and clones identified which carried
the insert fragment in the same orientation as the positive
(+) strand form of the single stranded mature phage.
Single-stranded phage DNA was prepared from one such clone
30 and designated m11VS α .

 To precisely remove codons 1-66 of v-sis, oligonu-
cleotide directed mutagenesis was performed essentially
according to the two primer method of Zoller (Zoller, et
al., Manual for Advanced Techniques in Molecular Cloning
35 Course, Cold Spring Harbor Laboratory, 1983). Oligonucleo-
tide ZC 130 (3' AGAAACCTATTTTCCTCGGACCCA 5') was
synthesized on an Applied Biosystems 380-A DNA synthesizer.

Fifty pmoles of ZC 130 were kinased in 10 ul of kinase buffer (BRL) with 4 units of T₄ polynucleotide kinase for 45 minutes at 37°C. The enzyme was inactivated by heating at 65°C for 10 minutes.

One-half pmole of mllVS α was annealed with 1 pmole of kinased ZC 130 and 1.5 pmoles of universal sequencing primer (BRL) using the conditions described (Zoller et al., *ibid.*), except that the annealing mixture was first heated to 65°C for 10 minutes, shifted to 37°C for 10 minutes, and then quickly chilled on ice. The annealed mixture was then treated with Klenow polymerase as described by Zoller et al. (*ibid.*) to create circular duplex DNA. Portions of the elongation mixture were used to transform E. coli K12 JM 101 cells. The resulting phage plaques were screened for the proper deletion by transfer onto nitrocellulose filters and subsequent hybridization with ³²P phosphorylated ZC 130 at 65°C. Correctly juxtaposed sequences formed stable duplexes with the radioactive probe at the stringent hybridization temperature employed. Approximately 1% of the transformants screened gave positive signals by autoradiography. Ten clones were plaque-purified and RF DNA was prepared for restriction enzyme analysis. Five isolates showed the expected decrease in size of 195 bp to the 1450 bp Hind III-Bgl II fragment (Figure 4). DNA sequence analysis of two isolates confirmed the correct fusion junction had been made, thus maintaining the proper translational reading frame. One of these phage was designated mllVS2 α .

30

EXAMPLE IV

Yeast Expression Vectors

A. Construction of Plasmids YEpVS α and YEpVS2 α .

35

Yeast replicating vector YEpl3 (Broach et al., Gene 8: 121, 1979) was used as an expression vehicle for

v-sis derived constructions described in Examples II and III. YEpl3 is a multicopy extrachromosomal plasmid containing a 2 micron replication origin and the yeast LEU2 gene. This allows for selection of the plasmid in yeast strains possessing a defective chromosomal LEU2 gene when grown on synthetic medium lacking leucine. Addition of yeast terminator sequences to foreign genes expressed in yeast ensures efficient transcription termination and polyadenylation of mRNA. The v-sis expression units VS α and VS2 α were placed adjacent to the TPI terminator fragment which was previously cloned into YEpl3 (below).

Plasmid p270 (see Figure 5) contains the transcription terminator region of the yeast triose phosphate isomerase (TPI) gene. It was constructed in the following manner. The yeast TPI terminator fragment was obtained from plasmid pFG1 (Alber and Kawasaki, *ibid.*). It encompasses the region from the penultimate amino acid codon of the TPI gene to the Eco RI site approximately 700 base pairs downstream. A Bam HI site was substituted for this unique Eco RI site of pFG1 by first cutting the plasmid with Eco RI, then blunting the ends with DNA polymerase I (Klenow fragment), adding synthetic Bam HI linkers (CGGATCCA), and re-ligating to produce plasmid p136. The TPI terminator was then excised from p136 as a Xba I-Bam HI fragment. This fragment was ligated into YEpl3 (Broach et al., *ibid.*) which had been linearized with Xba I and Bam HI. The resulting plasmid is known as p213. The Hind III site was then removed from the TPI terminator region of p213 by digesting the plasmid with Hind III, blunting the resultant termini with DNA polymerase I (Klenow fragment), and recircularizing the linear molecule using T₄ DNA ligase. The resulting plasmid is p270.

Alternatively, p270 may be constructed by digesting plasmid pM220 (see below) with Xba I and Bam HI, purifying the TPI terminator fragment (~700bp), and inserting this fragment into XbaI and Bam HI digested YEpl3.

Referring to Figure 6, plasmid p270 DNA was digested with Xba I and treated with calf alkaline phosphatase to prevent religation of the cohesive vector ends. V-sis expression units VS α and VS2 α were prepared by Xba I digestion and agarose gel purification of pVS α and mllvs2 α , respectively. Each of the isolated fragments was ligated with an approximately equimolar amount of phosphatased p270 vector in the presence of 40 units of T₄ DNA ligase and the ligation mixtures transformed into E. coli K-12 RR1. Plasmid DNA was prepared from ampicillin-resistant colonies and restriction enzyme analysis performed in order to identify clones which possessed the TPI terminator adjacent to 3' v-sis sequences. Presence of 3.3 kb or 3.1 kb Bgl II fragments after gel electrophoresis indicated the correct orientation of YE pVS α and YE pVS2 α , respectively.

B. Insertion of VS2 α Expression Unit into pCPOT.

In order to achieve maximal protein production from a yeast culture, it is desirable to use expression vehicles which are very stably maintained in the host cell. Plasmid pCPOT is such a preferred expression vehicle.

E. coli HB101 transformed with pCPOT has been deposited with American Type Culture Collection under accession number 39685. Plasmid pCPOT comprises the 2 micron circle genome (Hartley and Donelson, Nature 286: 860, 1980), E. coli plasmid pBR322 replication and selection sequences, and the Schizosaccharomyces pombe DNA sequences encoding the glycolytic enzyme triose phosphate isomerase (POT1). Presence of the POT1 gene in pCPOT ensures stable maintenance of the plasmid in the appropriate host background during growth on nonselective medium utilizing glucose as a carbon source.

The S. cerevisiae TPI promoter was used to control expression of VS2 α sequences in pCPOT. Plasmid pM220 contains the TPI promoter fused to the MF α 1 signal

sequence. E. coli RRI transformed with pM220 has been deposited with American Type Culture Collection under accession number 39853.

5 Referring to Figure 7, plasmid pM220 was digested with Bgl II and Bam HI, electrophoresed through a 0.9% agarose gel, and the 2.2 kb TPI promoter, MFol gene fragment extracted. The purified fragment was digested with Pst I and the resulting 1 kb Bgl II-Pst I fragment agarose
10 gel-purified as above. Plasmid YEpVS2 α was digested with Pst I and Bam HI, and the 1.8 kb MFol/v-sis/TPI terminator fusion fragment gel-isolated. Plasmid pCPOT was digested with Bam HI, treated with calf alkaline phosphatase, phenol/CHCl₃ extracted, then purified by electrophoresis
15 through agarose, extracted from the gel and EtOH precipitated.

Approximately equimolar amounts of the three isolated fragments described above (Figure 7) were ligated overnight at 12°C and the ligation mixture used to trans-
20 form E. coli K-12 strain DH1 (Hanahan, D. and Meselson, M., J. Mol. Biol. 166: 577, 1983) to ampicillin resistance. Plasmid DNA was prepared from transformants and restriction digest analysis used to ascertain the orientation of the insert fragments. Presence of the 1500 bp Bam HI-Sal I
25 fragment indicates that the Bam HI cohesive end of the TPI terminator fragment is oriented as shown in Figure 7. The opposite orientation would create a Bam HI/Bgl II fusion, not cleavable by Bam HI, and hence would not yield this fragment. The 800 bp Sph I fragment indicated that the TPI
30 promoter and v-sis fragments were properly fused at the Pst I site (Figure 7). This plasmid was designated pll7-2.

C. Construction of the Plasmid pVSB.

35 Because the product encoded by pVS2 α is larger than authentic human PDGF B chain and because a smaller product might result in higher expression levels in a

transformed yeast host cell, a vector was constructed comprising the v-sis sequence of pVS2 truncated at the 3' end. The polypeptide encoded by this sequence comprises
 5 amino acids 67 to 175 of p28^{Sis} and is homologous to the B chain of PDGF.

An expression vector containing this "B chain" sequence was constructed by combining elements of the pVS2 α expression unit with a partial v-sis gene and a synthetic
 10 double-stranded DNA fragment encoding amino acids 158 to 175 of p28^{Sis}. This synthetic fragment was designed to substitute preferred yeast codons for many of the 13 v-sis codons it replaces, and to supply a stop codon at the end of the coding sequence. The construction of this vector is
 15 illustrated in Figures 8 and 9.

Plasmid YE α pVS2 α was digested with Pst I and Bam HI and the 1.8 kb fragment comprising the partial M α F α I, v-sis, and TPI terminator sequences was purified by agarose gel electrophoresis. Plasmid pIC19R (obtainable from Dr.
 20 J. Lawrence Marsh, University of California, Irvine), comprising the polylinker shown in Chart 1 inserted into the Hind III site of pUC19 (Norrande et al., Gene 26: 101-106, 1983), was digested with Pst I and Bam HI, and the vector fragment was gel purified and joined to the 1.8 kb
 25 fragment from pVS2 α to produce plasmid pVS2 α T.

CHART 1

GAATTCATCGATATCTAGATCTCGAGCTCGCGAAAGCTT
 30 Eco RI Eco RV Bgl II Sac I Hind III
 Cla I Xba I Xho I Nru I

Plasmid pM220 was digested with Bgl II and Pst I, and the ca. 1 kb fragment comprising the TPI promoter and the 5'
 35 portion of the M α F α I sequence was isolated and cloned in Bgl II + Pst I digested pIC19R. The resultant plasmid was digested with Cla I and Pst I, and the TPI promoter - M α F α I

fragment was gel purified. Plasmid pVS2 α T was then cut with Cla I and Pst I and joined to the TPI promoter - MF α 1 fragment. The correct construct was identified by the
 5 presence of a 2.6 kb Cla I - Bam HI fragment and was designated pTVS2 α T.

Ten μ g of plasmid pVS α was digested with Xma I and Sph I to completion. The resulting ca. 4.9 kb vector fragment, which also comprises most of the v-sis sequence,
 10 was purified by agarose gel electrophoresis, extraction of the DNA and EtOH precipitation.

In order to supply a new 3' terminus for the v-sis sequence, a double-stranded DNA fragment was constructed from oligonucleotides synthesized on an Applied
 15 Biosystems Model 380-A DNA synthesizer. 0.7 pmole of oligonucleotide ZC299 (Table 1) was heated with an equimolar amount of oligonucleotide ZC300 in a volume of 10 μ l containing 40 mM NaCl for 5 minutes at 65°C.

20

TABLE 1

ZC299: 5'TAAG TGT GAA ATC GTT GCC GCG GCT AGA GCT GTT ACC
 TAA TCT AGA^{3'}

25 ZC300: 3'GTACA TTC ACA CTT TAG CAA CGG CGC CGA TCT CGA CAA
 TGG ATT AGA TCT GGCC^{5'}

The mixture was then incubated at 37°C for 5 minutes and allowed to cool to room temperature. 0.2 pmole of the purified
 30 4.9 kb vector fragment was added, the mixture ligated for 18 hours at 12°C and used to transform E. coli HB101 (ATCC 33694) to ampicillin resistance. DNA was prepared from ampicillin-resistant colonies and digested with Bgl II and Xba I. After electrophoresis through agarose, the
 35 desired clone (known as pVS α B) was identified by loss of a ca. 750 bp Bgl II--Xba I fragment and appearance of two smaller fragments of approximately 500 and 260 bp.

Approximately 8 ug of plasmid pTVS2 α T was digested to completion with Xba I in a volume of 10 ul. The volume was increased to 40 ul with Bgl II buffer, and 6 units of Bgl II were added and the mixture was incubated at 37°C. Ten ul aliquots were removed to a stop buffer containing 50 mM EDTA at 15 and 30 minutes, and the remaining 20 ul stopped at 45 minutes. The resulting mixtures were separated by electrophoresis through 0.7% agarose. The ca. 4.6 kb Bgl II--Xba I vector fragment was cut out, extracted from the gel, and EtOH precipitated. Plasmid pVS α B was digested with Bgl II and Xba I, and the ca. 260 bp fragment containing the synthetic 3' terminus and stop codon was isolated by electrophoresis through agarose, subsequent extraction from the gel, and EtOH precipitation.

The 4.6 kb Bgl II-Xba I vector fragment from pTVS2 α T and the 260 bp Bgl II--Xba I fragment from pVS α B were ligated in the presence of T4 DNA ligase for 7 hours at room temperature. The reaction mixture was used to transform E. coli HB101 to ampicillin resistance. DNA was prepared from transformants and the presence of the desired insert was confirmed by screening for a 550 bp Pst I--Xba I band on an agarose gel. A plasmid having the correct configuration was designated pVSB.

25

D. Construction of pMPOT2.

For expression of the v-sis derivations in yeast, a stable expression vector comprising the REP1, REP2, REP3, and ori sequences from yeast 2 micron DNA and the Schizosaccharomyces pombe triose phosphate isomerase (POT1) gene was constructed. The POT1 gene provides for plasmid maintenance in a transformed yeast host grown in complex media if such host is defective for triose phosphate isomerase.

The POT1 gene was obtained from the plasmid pFATPOT. S. cerevisiae strain El8 transformed with pFATPOT has been deposited with ATCC under accession number 20699.

The plasmid may be purified from the host cells by conventional techniques. The POT1 sequence was removed from pFATPOT by digestion of the plasmid with Sal I and Bam HI. 5 This ~1600 bp fragment was then ligated to pIC19R, which had first been linearized by digestion with Sal I and Bam HI. The Bam HI, Pst I and Sal I sites in the resultant plasmid were destroyed in two steps to produce plasmid pICPOT*. The Pst I and Sal I sites were removed by cutting 10 with Pst I and Sal I; the ends were blunted by digesting the Pst I 3' overhang with DNA polymerase I (Klenow fragment) and filling in the Sal I 5' overhang with Klenow fragment. The blunt ends were then ligated. The Bam HI site was then removed by cutting the plasmid with Bam HI, 15 filling in the ends with DNA polymerase I (Klenow fragment) and religating the blunt ends.

The 2u sequences were obtained from the plasmids YEpl3 (Broach et al., Gene 8: 121-133, 1979) and Cl/1. Cl/1 was constructed from pJDB248 (Beggs, Nature 275: 20 104-109, 1978) by removal of the pMB9 sequences by partial digestion with Eco RI and replacement by Eco RI-cut pBR322. The REP3 and ori sequences were removed from YEpl3 by digestion with Pst I and Xba I and gel purification. REP2 was obtained from Cl/1 by digestion with Xba I and Sph I and 25 gel purification. The two fragments were then joined to pUC18 (Norrande et al., Gene 26: 101-106, 1983) which had been linearized with Pst I and Sph I to produce plasmid pUCREP2,3. REPl was obtained from Cl/1 by digestion with Eco RI and Xba I and gel purification of the 1704 bp frag- 30 ment. The Eco RI--Xba I fragment was cloned into pUC13 which had been linearized with Eco RI and Xba I. The resultant plasmid was designated pUC13 + REPl. The pUC13 + REPl plasmid was cut with Hind II and ligated in the presence of Eco RI linkers (obtained from Bethesda Research 35 Laboratories). The REPl gene was then removed as an Eco RI fragment of approximately 1720 bp. This Eco RI fragment was cloned into pIC7 (comprising the polylinker sequence

shown in Chart 1 inserted into the Hind III site of pUC8), which had been linearized with Eco RI and Xba I. The resultant plasmid was designated pICREPl#9.

5 To construct the final expression vector pMPOT2, pICPOT* was linearized by a partial Hind III digestion and complete Sst I digestion. Plasmid pUCREP2,3 was cut with Hind III and Sst I, and the fragment comprising REP2, REP3 and ori sequences was gel purified and joined to the linear-
10 ized pICPOT*. The resultant plasmid, comprising REP2, REP3, ori, POT1 and amp^r sequences, was designated pMPOT1. REP1 was then removed from pICREPl #9 as a Bgl II--Nar I fragment and was ligated to pMPOT1, which had been cleaved with Bgl II and Nar I. The product of this ligation was
15 designated pMPOT2 (deposited with ATCC, accession number 20744). Plasmid pMPOT2 was digested with Cla I and Bam HI, and the vector fragment was purified as above.

E. Insertion of v-sis Expression Units in pMPOT2.

20

1. Insertion of VS α expression unit into pMPOT2.

Approximately 10 ug of plasmid pVS α was digested with Bst EII to completion in a volume of 20 ul. Five
25 units of Pst I were added, the mixture was incubated 30 minutes and the reaction stopped by the addition of EDTA. The quenched reaction mixture was immediately electrophoresed through a 1% agarose gel, and the ca. 800 bp partial Pst I--Bst EII band (comprising most of the MF α 1 prepro
30 sequence and the 5' portion of v-sis) was cut out, extracted from the gel, and EtOH precipitated.

Plasmid pTVS2 α T was digested to completion with Pst I and Bst EII and purified by agarose gel electrophoresis. The resulting ca. 4.8 kb vector fragment and the
35 800 bp Pst I--Bst EII fragment were ligated in the presence of T₄ DNA ligase for 6 hours at room temperature, and the ligation mixture was used to transform E. coli HB101 to

ampicillin resistance. A plasmid was identified which contained a ca. 1450 bp Bgl II fragment, which indicated the presence of the insert. It was designated pTVS α .

5 Plasmid pTVS α was digested to completion with Cla I and Bam HI, and the ca. 2.9 kb fragment containing VS α sequences was isolated by electrophoresis through agarose, extraction from the gel, and EtOH precipitation. The ca. 2.9 kb Cla I--Bam HI VS fragment was ligated with Cla I
10 and Bam HI digested pMPOT2 as described for pVS2 α m (below). A plasmid containing a 2.9 kb Cla I--Bam HI insert was identified and designated pVS α m.

2. Insertion of VS2 α expression unit into MPOT2.

15

Plasmid pTVS2 α T was digested to completion with Cla I and Bam HI in Bam HI buffer. The buffer was adjusted to high salt (Maniatis et al, ibid.) and the DNA was digested to completion with Pvu I, which cuts the vector
20 Cla I--Bam HI fragment containing the VS2 α sequences on an agarose gel. This fragment was electrophoresed through 0.9% agarose, extracted, and EtOH precipitated. The fragment was then ligated with Cla I--Bam HI digested pMPOT2 in
25 the presence of T4 DNA ligase for 20 hours at 13°C. The ligated DNA was used to transform E. coli HB 101 to ampicillin resistance, and plasmid DNA was prepared from the resulting colonies. A plasmid was identified which contained the 2.7 kb Cla I--Bam HI VS2 α fragment and was
30 designated pVS2 α m.

3. Insertion of VSB expression unit into pMPOT2.

35 Plasmid pVSB was digested with Cla I and Bam HI, and the 2.2 kb fragment containing the "B chain" expression unit purified by agarose gel electrophoresis and EtOH precipitation. The fragments were ligated overnight at room

temperature in the presence of T4 DNA ligase and the reaction mixture used to transform E. coli HB101 to ampicillin resistance. DNA was prepared from transformants and the presence of the insert verified by digestion with Cla I and Bam HI and agarose gel electrophoresis. The resulting expression vector was designated pVSBm.

10

EXAMPLE V

Yeast Transformation; and Analysis of v-sis Transcription

S. cerevisiae strain E8-11c (MAT α leu2-3, 112 pep 4-3; a haploid segregant of the cross E2-7B [ATCC 20689] x GK 100 [ATCC 20689]) was transformed with plasmids YEpVS α , YEpVS2 α , p270, pl17-2 and pCPOT. Transformants were selected and maintained in synthetic medium lacking leucine.

20

S. cerevisiae strain E11-3c (ATCC Accession #20727) (MAT α pep4-3 tpil) was transformed with plasmids pCPOT and pl17-2. Transformants were selected and maintained in YEPD.

Referring to Figure 8, presence of v-sis related mRNA transcripts was confirmed by electrophoretic and subsequent hybridization analysis of total RNA. Total RNA from the above-described transformants in strain E8-11c was prepared by guanidinium thiocyanate extraction as described by Maniatis et al. (ibid.) with the following modifications: 100ml cultures were grown to a density of 1×10^8 cells/ml. The cells were pelleted by centrifugation and washed three times with H₂O, 2 mls of guanidinium lysis solution was added, followed by 0.5mm glass beads to just below the meniscus. The tubes were vortexed three times for 1 minute, with cooling on ice between bursts. The solution was pipetted off and the RNA isolated by centrifugation through CsCl₂ as described (Maniatis et al., ibid.).

Fifteen ug of RNA from plasmid transformants p270, YEpVS α , YEpVS2 α , pCPOT and pll7-2 was glyoxylated, electrophoresed through a 0.9% agarose gel and transferred to nitrocellulose as described by Thomas (PNAS 77: 5201, 1980). The purified Pst I v-sis fragment from pVSIS/Pst was nick-translated and hybridized to the filter bound RNA, and the hybridizing species detected by autoradiography (Figure 10). Transcript bands of ~1900 bp from YEpVS α , ~1650 bp from YEpVS2 α , and ~1700 bp from pll7-2 confirmed the transcription of the v-sis fusion constructs and the use of the transcription start and stop signals in the constructions. No v-sis related transcripts were detected in negative controls p270 and pCPOT.

Plasmids pVS α m, pVS2 α m, pVSBm, and pMPOT2 were used to transform S. cerevisiae strain E18. Strain E18 is a diploid produced by crossing strains Ell-3c (ATCC No. 20727) and Δ tpi 29. Δ tpi 29 is produced by disrupting the triose phosphate isomerase gene of strain E2-7B (ATCC No. 20689), essentially as described by Rothstein (Meth. in Enzymology 101: 202-210, 1983).

EXAMPLE VI

Analysis of sis-related Products Expressed by Yeast; and
Biological Activity Assays

A. Concentration of Yeast Culture Medium.

Transformants carrying YEpl3 and pCPOT derived v-sis constructions were grown in the appropriate media at 30°C (1.2 liter cultures) to stationary phase on a rotary air shaker with agitation at 220 rpm. Cultures were harvested, the cells removed by centrifugation, and the medium concentrated on a C-8 Sepharose (Pharmacia Fine Chemicals AB, Uppsala, Sweden) column which binds molecules of a hydrophobic nature. Authentic human PDGF is a highly cationic and hydrophobic protein (Heldin et al., PNAS 76:

3722, 1979; Raines and Ross, *ibid.*). The sis-related putative yeast product was expected to possess similar characteristics. The sis product's expected hydrophobic character was exploited to concentrate it from the yeast media into which it was expected to be secreted. Molecules bound to the C-8 column were eluted from the matrix with suitable hydrophobic solvents.

Spent growth media from the transformed yeast cultures was adjusted to 5% EtOH and passed through an 8 ml C-8 Sepharose column at a flow rate of 2-3 ml per minute. The column was then washed with 100mls of 5% EtOH in 20 mM ammonium bicarbonate (NH_4HCO_3). The bound material was eluted with 20% propanol in 20mM NH_4HCO_3 and the eluate collected in 1-2 ml fractions. Fractions were assayed for protein content by light absorption at 280 nm, (A_{280} of 1.4=1.0 mg protein/ml) or by the method of Lowry et al. (J. Biol. Chem 193: 265, 1951). The concentrated fractions were combined, lyophilized, and then resuspended in 500-700 ul of PBS (phosphate buffered saline, pH 7.4).

Transformant pll7-2 in strain Ell-3c grown under POT1 selection (with glucose as carbon source) was expected to produce significantly higher levels of PDGF-like material in the media and thus was analyzed after dialysis of the media against PBS without concentration.

Media samples from the transformants pVS α m, pVS2 α m, pVSBm and pMPOT2 were concentrated by adsorption to CM-sephadex and elution with 1M NaCl in 1M acetic acid, pH 4.5. The concentrated media were dialyzed against 0.1 M acetic acid, pH 7 and the amount of PDGF-like material in the concentrates was determined by ELISA.

B. Detection of PDGF-like Material by Enzyme-Linked Immunosorbent Assay (ELISA)

35

The expression of PDGF-like molecules by the yeast transformants was examined by ELISA and quantitated

by comparison to a standard curve developed with purified human PDGF (Raines and Ross, *ibid.*). A typical standard curve was prepared as follows:

5 Purified human PDGF, 2.5 ng/ml in PBS, was incubated overnight with Immulon II (Dynatech Laboratories, Inc.) 96 well microtiter plates (100 ul/well) at 4°C. This coating solution was removed and 100 ul/well of 0.1% rabbit albumin in PBS was added and the plates incubated for 1 hour at
10 37°C. Samples of purified PDGF (0.1-40ng/ml) were separately incubated with goat anti-PDGF IgG (5 ug/ml) in PBS containing 0.05% Tween 20 and 1 mg/ml rabbit albumin (RSA). The microtiter plates were washed 5 times with 0.9% NaCl, .05% Tween 20, drained, and 100 ul of each test solution
15 was added to the microtiter wells and incubated 2 hours at 37°C. The plates were washed as before, and peroxidase-conjugated swine anti-goat IgG (Tago, Inc.) diluted 1:1000 in PBS containing 0.05% Tween 20 and 1 mg/ml RSA was added for 2 hours at 37°C. The plates were washed as before and
20 freshly prepared .04% o-phenylene diamine containing .012% hydrogen peroxide (H₂O₂) (100 ul/well) was added for 50 minutes at room temperature and the reaction stopped at 50 minutes by the addition of 4N H₂SO₄ (50 ul/well). Absorbance at 492 nm was determined using a Dynatech plate
25 scanner. Each test point was measured in triplicate and plotted as the mean \pm standard error. C-8 eluates of yeast culture media and unconcentrated media samples were diluted in PBS, assayed as described and compared to the PDGF standard curve. Table 2 is a summary of assay results for
30 a representative series of experiments. Figure 11 depicts an ELISA of a range of C-8 eluate sample volumes measured, generating a dose-response curve which is compared to a standard curve from purified PDGF.

Raw ELISA data for the MPOT constructions are not
35 shown, but have been incorporated into the radioreceptor and mitogenesis assay data as shown in Figures 13 and 14.

C. Radioreceptor Assay (RRA) for PDGF.

The radioreceptor assay for PDGF (Bowen-Pope and
5 Ross, J. Biol. Chem 257: 5161, 1982) is a specific and
sensitive (.2-2 ng/ml PDGF) method for detecting biological-
ly active PDGF-like material in yeast. In this assay,
PDGF-like material is tested for its ability to compete
with purified, radio-labeled ^{125}I PDGF for binding sites on
10 cell surface PDGF receptors. Results are interpreted by
comparison to a standard curve generated with purified,
unlabeled PDGF. Comparison of results obtained with other
assay methods (e.g., ELISA) provides an indication of the
strength of the receptor/ligand interaction in addition to
15 quantitation of the material bound. The assay is conducted
as follows: Subconfluent monolayers of diploid human
fibroblasts are prepared by plating 1.5×10^4 cells per 2cm^2
culture well in Costar 24 well cluster trays in Dulbeccos
Modified Eagles Medium (DMEM) supplemented with 1% human
20 plasma-derived serum (PDS). Cultures are set on an ice
tray and rinsed once with ice-cold binding rinse (Ham's
medium F-12 buffered at pH 7.4 with 25mM HEPES and supple-
mented with 0.25% BSA). One ml/well of test substance in
binding medium is added and the cultures incubated in a
25 refrigerated room on an oscillating platform for 3-4 hours.
The trays are then placed on ice, aspirated, rinsed once
with cold binding rinse, and incubated for one hour as
above with 1 ml/well binding medium containing 0.5 ng/ml
 ^{125}I -PDGF. Labeling is terminated with 4 rinses of binding
30 rinse and cell-associated ^{125}I -PDGF determined by extrac-
tion with solubilization buffer. Standard curves are
obtained using 0, 0.05, 0.1, 0.2, 0.4, and 0.8 ng/ml
purified PDGF and test samples compared to these values.

Results obtained by RRA for yeast C-8 eluates and
35 1X media samples are given in Table 2.

In addition, PDGF receptor binding by CM-sepha-
dex media concentrates from yeast transformants containing

plasmids pVS α m, pVS2 α m, pVSBm, and pMPOT2 was compared to authentic PDGF. The results were interpreted by comparison to a standard curve generated with purified, unlabeled PDGF, as shown in Figure 13. Media from cultures transformed with the v-sis constructions are shown to compete with ^{125}I -PDGF for binding to the PDGF receptor. Media from yeast cells transformed with pMPOT2 do not compete with radio-labeled PDGF for receptor binding.

10

D. Mitogenesis Assay.

The ability of PDGF to stimulate DNA synthesis and cell growth in culture was the basis for its definition and discovery. ^3H -Thymidine incorporation into DNA of cultured cells responsive to PDGF (Raines and Ross, Meth. in Enzymology 109: in press) is a preferred method for demonstrating the biological activity of PDGF-like molecules produced in yeast.

20

Test samples in 10mM acetic acid (100 μl /well) are added to quiescent cultures of mouse 3T3 cells in 2cm² Costar 24-well culture dishes (2-3x10⁸ cells/well in 1 ml). Quiescent test cultures can be obtained by plating the cells in 10% serum and allowing them to deplete the medium, 4-5 days. The test samples are removed from the wells at 20 hours and replaced with 0.5 ml of fresh medium per well containing 2 $\mu\text{Ci/ml}$ [^3H]-Thymidine and 5% (v/v) calf serum. After an additional 2-hour incubation at 37°C the cells are harvested by: aspirating off the medium, washing the wells twice each with 1 ml of ice-cold 5% TCA; solublizing TCA-insoluble material in 0.8 ml 0.25N NaOH with mixing; and counting 0.6 ml of this solution in 5 ml Aquasol in a liquid scintillation counter. Fold stimulation over control wells (100 μl of 10mM acetic acid alone) is determined (normally 30-50 fold maximal stimulation) and compared to a standard curve obtained using purified PDGF preparations.

35

Table 2 presents results obtained in the mitogenesis assay for PDGF-like material produced in yeast and compares the activities of the PDGF-like material as measured by the above-described assay methods. Figure 12 depicts the mitogenic response elicited by concentrated media from pll7-2 transformed Ell-3c and pVS α transformed E8-11c compared to that obtained with purified human PDGF.

10

TABLE 2

<u>Preparation</u>	<u>Transformant</u>	<u>ug/ml</u> <u>Protein</u>	<u>ng/ml PDGF by</u>			
			<u>ELISA</u>	<u>RRA</u>	<u>MITOGENESIS</u>	
15	C-8 Eluates					
	pVS α /E8-11c	2.00	188	4.6	102	
	pVS2 α /E8-11c	16.00	864	16-97	310	
	pl17-2/E11-3c	1.44	120	13.9	87	
20	1X Media	pl17-2/E11-3c	--	4.2	0.18	2.5

20

In addition, the mitogenic response elicited by CM-sephadex concentrates from yeast transformants containing plasmids pVS α m, pVS2 α m, pVSBm, and pMPOT2 was compared to that obtained with authentic PDGF. Referring to Figure 14, media from cultures transformed with the v-sis constructions stimulated uptake of 3 H-thymidine by quiescent 3T3 cells. As noted above, uptake of 3 H-thymidine by quiescent 3T3 cells is taken to be indicative of mitogenic stimulation. Media from yeast cells transformed with pMPOT2 showed no mitogenic activity.

30

The data present clear evidence that growth media from the yeast strains constructed herein possess biological activities identical to authentic human PDGF. Further, these activities are readily detectable in nonconcentrated

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(1X) media from p117-2 transformed strain Ell-3c grown under POT1 selection.

5 The VSB preparation was further characterized by SDS-polyacryamide gel electrophoresis and subsequent mitogenesis assay of gel fractionated material. Ninety percent of the VSB mitogenic activity is recovered from the 30 kilodalton region of the gel (Figure 15). This indicates that this expression unit produces a very homogeneous and likely
10 fully processed material. In this sense, the VSB protein is superior to the VS2 α and VS α proteins described above, which are quite heterogeneous. In addition, the VSB material is a positive chemoattractant, indicating that it may possess the full spectrum of PDGF biological activities.

15 From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is
20 not limited except as by the appended claims.

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Claims

1. A DNA construct capable of directing the expression and secretion of biologically active PDGF analogs in eucaryotic cells, said DNA construct containing a transcriptional promoter followed downstream by a gene encoding a protein having substantially the same structure and/or mitogenic activity as PDGF, and a signal sequence directing the secretion of the protein from the eucaryotic cell.

2. The DNA construct of claim 1 wherein the eucaryotic cell is a yeast cell, and the promoter and signal sequence are of yeast origin.

3. The DNA construct of claim 1 wherein said gene is the v-sis gene of simian sarcoma virus or a derivative thereof encoding a protein having biological activity.

4. The DNA construct of claim 3 wherein the derivative of the v-sis gene of simian sarcoma virus is the portion of the v-sis gene encoding a protein substantially homologous to the B chain of PDGF.

5. The DNA construct of claim 1 wherein said gene is the human cDNA gene for PDGF or portions thereof encoding a protein having biological activity.

6. A method of preparing biologically active PDGF analogs, comprising:

introducing into a eucaryotic host cell a DNA construct capable of directing the expression and secretion of biologically active PDGF analogs in eucaryotic cells, said DNA construct containing a transcriptional promoter followed downstream by a gene encoding a protein having substantially the same structure and/or mitogenic activity as PDGF, and a signal

sequence capable of directing the secretion of the protein from the eucaryotic host cell;

growing said eucaryotic host cell in an appropriate medium; and

isolating the protein product of said gene from said eucaryotic host cell.

7. The method of claim 6 wherein the eucaryotic cell is a yeast cell, and the promoter and signal sequence are of yeast origin.

8. The method of claim 6 wherein said gene is the v-sis gene of simian sarcoma virus or a derivative thereof encoding a protein having biological activity.

9. The method of claim 8 wherein the derivative of the v-sis gene of simian sarcoma virus is the portion of the v-sis gene encoding a protein substantially homologous to the B chain of PDGF.

10. The method of claim 6 wherein said gene is the human cDNA gene for PDGF or portions thereof encoding a protein having biological activity.

11. Biologically active PDGF analogs obtainable by a method as claimed in any one of claims 6-10.

12. A eucaryotic cell transformed with a DNA construct as claimed in any one of claims 1-5.

13. The eucaryotic cell of claim 12 wherein said DNA construct is the plasmid pVSBm.

14. A method of promoting the growth of mammalian cells, comprising incubating the cells with a biologically active PDGF analog expressed by a eucaryotic cell transformed with a DNA construct as claimed in any one of claims 1-5.

15. A DNA construct capable of replication in yeast and containing the yeast triose phosphate isomerase promoter, said yeast promoter being followed downstream by the signal sequence of the gene encoding the yeast mating pheromone alpha-factor, said signal sequence being followed downstream respectively by the portion of the v-sis gene encoding a protein substantially homologous to the B chain of PDGF and the yeast triose phosphate isomerase terminator.

16. The plasmid pVSBm.

17. A DNA construct capable of directing the expression and secretion of biologically active PDGF analogs in eucaryotic cells, said DNA construct containing a transcriptional promoter followed downstream by a DNA sequence encoding a protein substantially homologous to the B chain of PDGF, said protein having substantially the same structure and/or mitogenic activity as PDGF, and a signal sequence directing the secretion of the protein from the eucaryotic cell.

18. A method of preparing biologically active PDGF analogs, comprising:

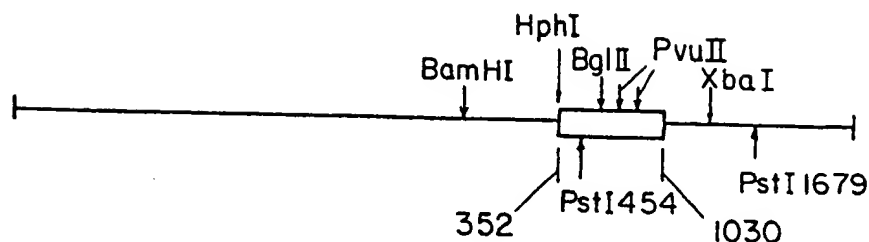
introducing into a eucaryotic host cell a DNA construct capable of directing the expression and secretion of biologically active PDGF analogs in eucaryotic cells, said DNA construct containing a transcriptional promoter followed downstream by a DNA sequence encoding a protein which is substantially homologous to the B chain of PDGF, said protein having substantially the same structure and/or mitogenic activity as PDGF, and a signal sequence directing the secretion of the protein from the eucaryotic host cell;

growing said eucaryotic host cell in an appropriate medium; and

isolating the protein product of said gene from said eucaryotic host cell.

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FIG. 1A



Hph I
 CT ATG ACC CTC ACC TGG CAG GGG GAC CCC ATT CCT GAG GAG CTC TAT AAG ATG
 MET Thr Leu Thr Trp Gln Gly Asp Pro Ile Pro Glu Glu Leu Tyr Lys MET

367 382 397
 v-sis-helper viral junction

412 427 442 Pst I
 CTG AGT GGC CAC TCG ATT CGC TCC TTC AAT GAC CTC CAG CGC CTG CTG CAG GGA
 Leu Ser Gly His Ser Ile Arg Ser Phe Asn Asp Leu Gln Arg Leu Leu Gln Gly

472 487 502
 GAG TCC GGA AAA GAA GAT GGG GCT GAG CTG GAC CTG AAC ATG ACC CGC TCC CAT
 Asp Ser Gly Lys Glu Asp Gly Ala Glu Leu Asp Leu Asn MET Thr Arg Ser His

517 532 547 562
 TCT GGT GGC GAG CTG GAG AGC TTG GCT CGT GGG AAA AGG AGC CTG GGT TCC CTG
 Ser Gly Gly Glu Leu Glu Ser Leu Ala Arg Gly Lys Arg Ser Leu Gly Ser Leu

577 592 607
 AGC GTT GCC GAG CCA GCC ATG ATT GCC GAG TGC AAG ACA CGA ACC GAG GTG TTC
 Ser Val Ala Glu Pro Ala MET Ile Ala Glu Cys Lys Thr Arg Thr Glu Val Phe

Bgl II
 622 637 652 667
 GAG ATC TCC CGG CGC CTC ATC GAC CGC ACC AAT GCC AAC TTC CTG GTG TGG CCG
 Glu Ile Ser Arg Arg Leu Ile Asp Arg Thr Asn Ala Asn Phe Leu Val Trp Pro

FIG. 1B

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682 697 712 727
 CCC TGC GTG GAG GTG CAG CGC TGC TCC GGC TGT TGC AAC AAC CGC AAC GTG CAG
 Pro Cys Val Glu Val Gln Arg Cys Ser Gly Cys Cys Asn Asn Arg Asn Val Gln

Pvu II
 742 757 772
 TGC CGG CCC ACC CAA GTG CAG CTG CGG CCA GTC CAG GTG AGA AAG ATC GAG ATT
 Cys Arg Pro Thr Gln Val Gln Leu Arg Pro Val Gln Val Arg Lys Ile Glu Ile

787 802 817 832
 GTG CGG AAG AAG CCA ATC TTT AAG AAG GCC ACG GTG ACG CTG GAG GAC CAC CTG
 Val Arg Lys Lys Pro Ile Phe Lys Lys Ala Thr Val Thr Leu Glu Asp His Leu

Pvu II
 847 862 877
 GCA TGC AAG TGT GAG ATA GTG GCA GCT GCA CGG GCT GTG ACC CGA AGC CCG GGG
 Ala Cys Lys Cys Glu Ile Val Ala Ala Arg Ala Val Thr Arg Ser Pro Gly

892 907 922 937
 ACT TCC CAG GAG CAG CGA GCC AAA ACG ACC CAA AGT CGG GTG ACC ATC CGG ACG
 Thr Ser Gln Glu Gln Arg Ala Lys Thr Thr Gln Ser Arg Val Thr Ile Arg Thr

952 967 982 997
 GTG CGA GTC CGC CGG CCC CCC AAG GGC AAG CAC CGG AAA TGC AAG CAC ACG CAT
 Val Arg Val Arg Arg Pro Pro Lys Gly Lys His Arg Lys Cys Lys His Thr His

1012 1027 1043 1053
 GAC AAG ACG GCA CTG AAG GAG ACC CTC GGA GCC TAA GGGCATCGGC AGGAGAATAT
 Asp Lys Thr Ala Leu Lys Glu Thr Leu Gly Ala

1063 1073 1083 1093 1103 1113 1123
 GGGCAGCGGG TCTCCTGCCA GCGGCCTCCA GCATCTTGCC CAGCAGCTCA AGAAGAGAAA AAAGGACTGA

1133 1143 1153 1163 1173 1183 1193
 ACTCCACCAC CATCTTCTTC CCTTAAGTCC AAAAAGTTGA AATAAGAGTG TGAAAGAGAC TGATAGGGTC

1203 1213 1223 1233 1243 1253 1263
 GCTGTTTGAA AAAAAGTGGC TCCTTCCTCT GCACCTGGCC TGGGCCACAC CCAAGTGCTG TGGACTGGCC

1273 1283 1293 1303 1313 1323 1333
 CGAGGGGGCC TGCACGTGGC CCTGAGCACC TCTCAGTGTA GCCTGCCTGG TCCCTAGACC CCTGGCCAGC

1343 1353 1363 1373 XbaI v-sis-helper viral junction
 TCCAAGGGGA GGCACCTCCA GGCAGGCCAG GCTACCTCGG GGGTCTAG

FIG. 1B

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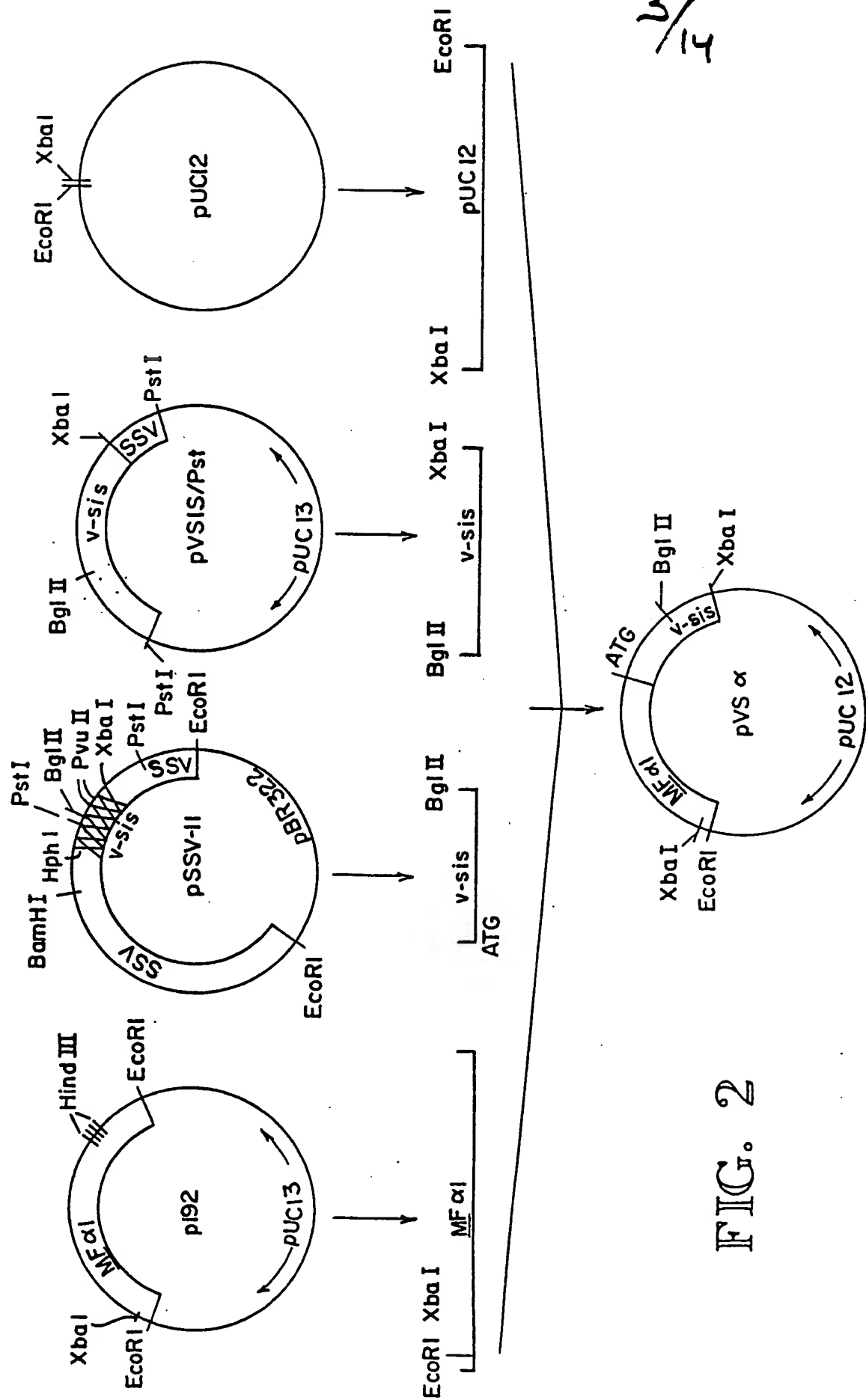
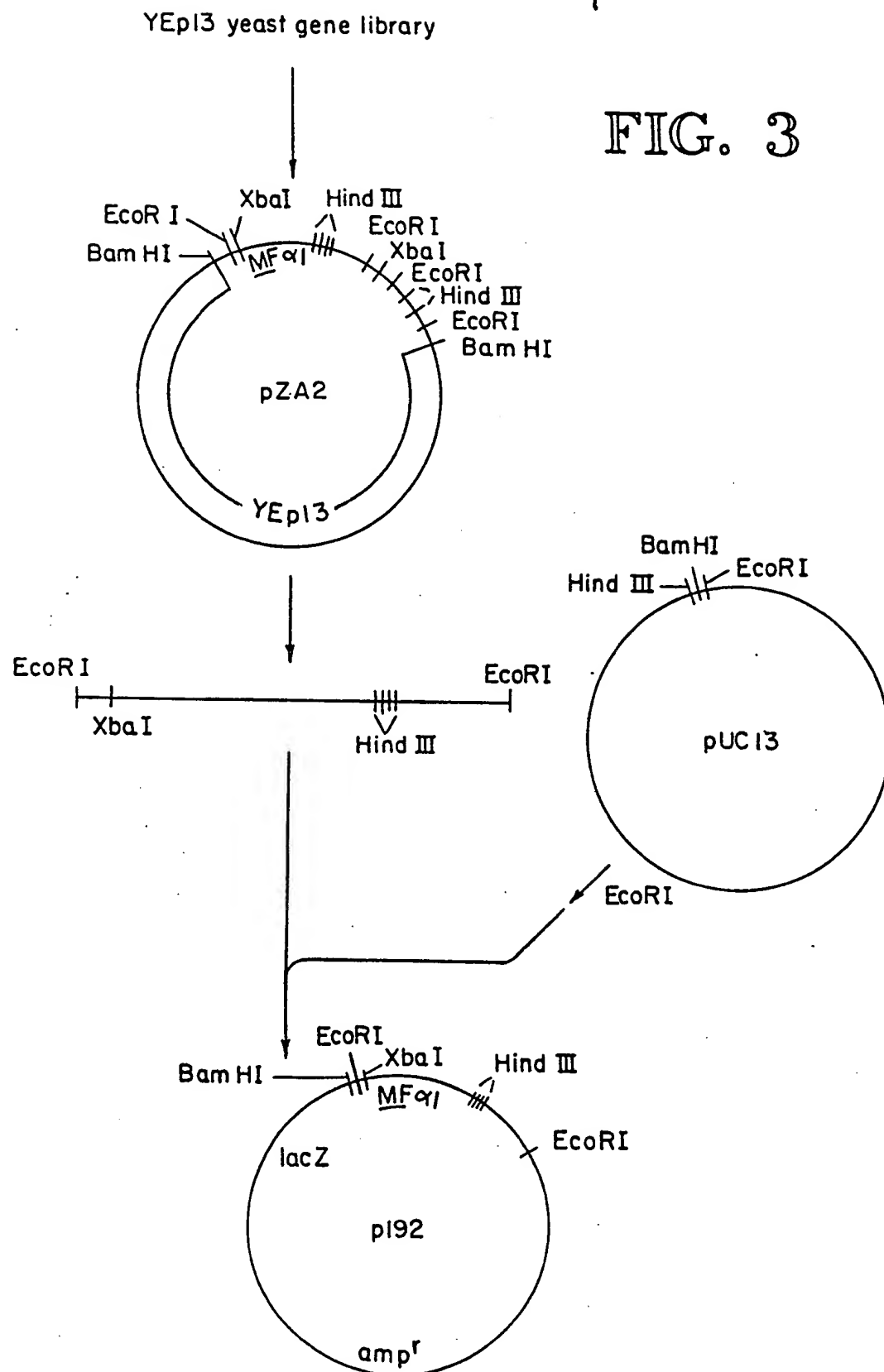


FIG. 2

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FIG. 3



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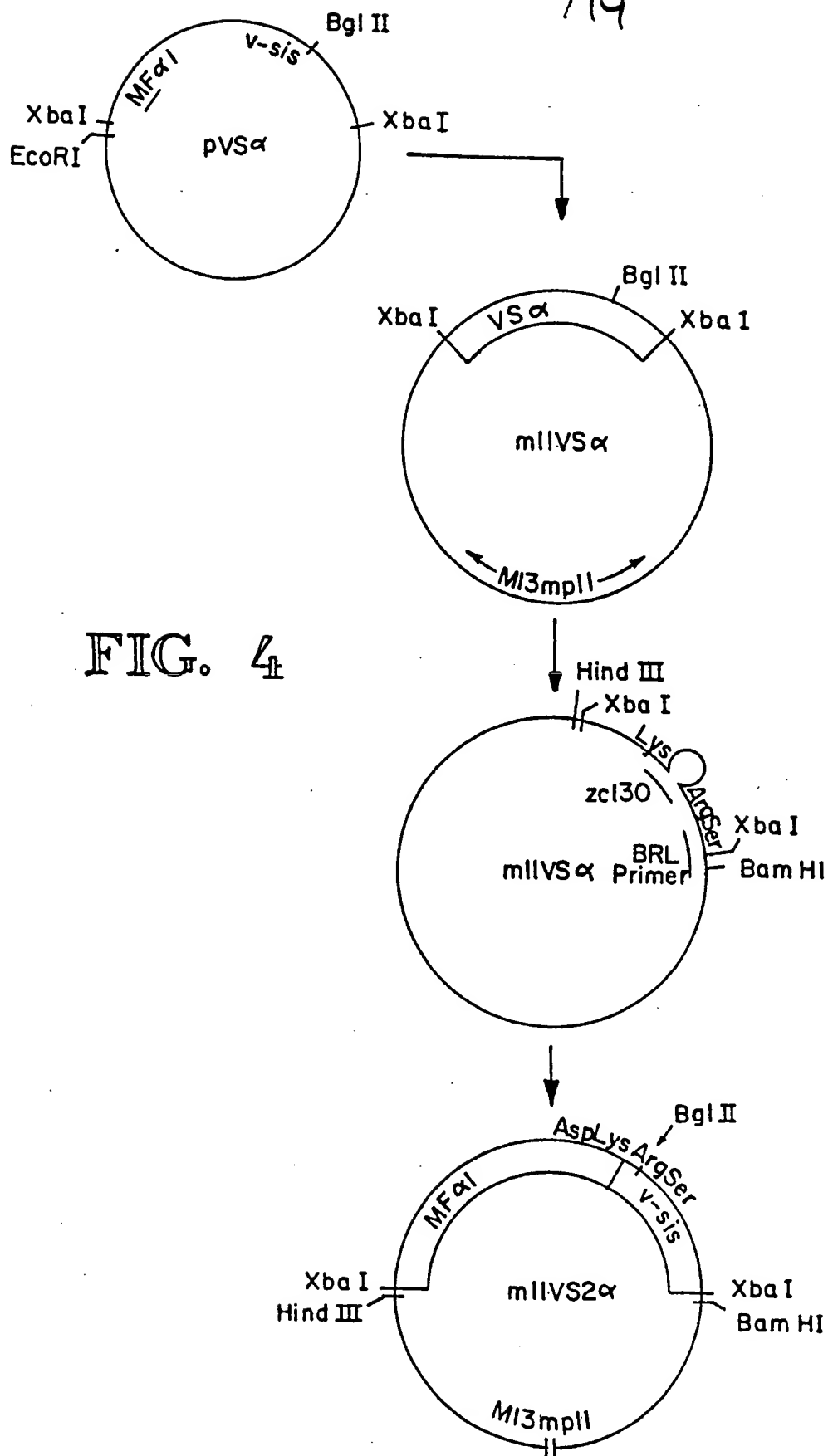


FIG. 4

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FIG. 5

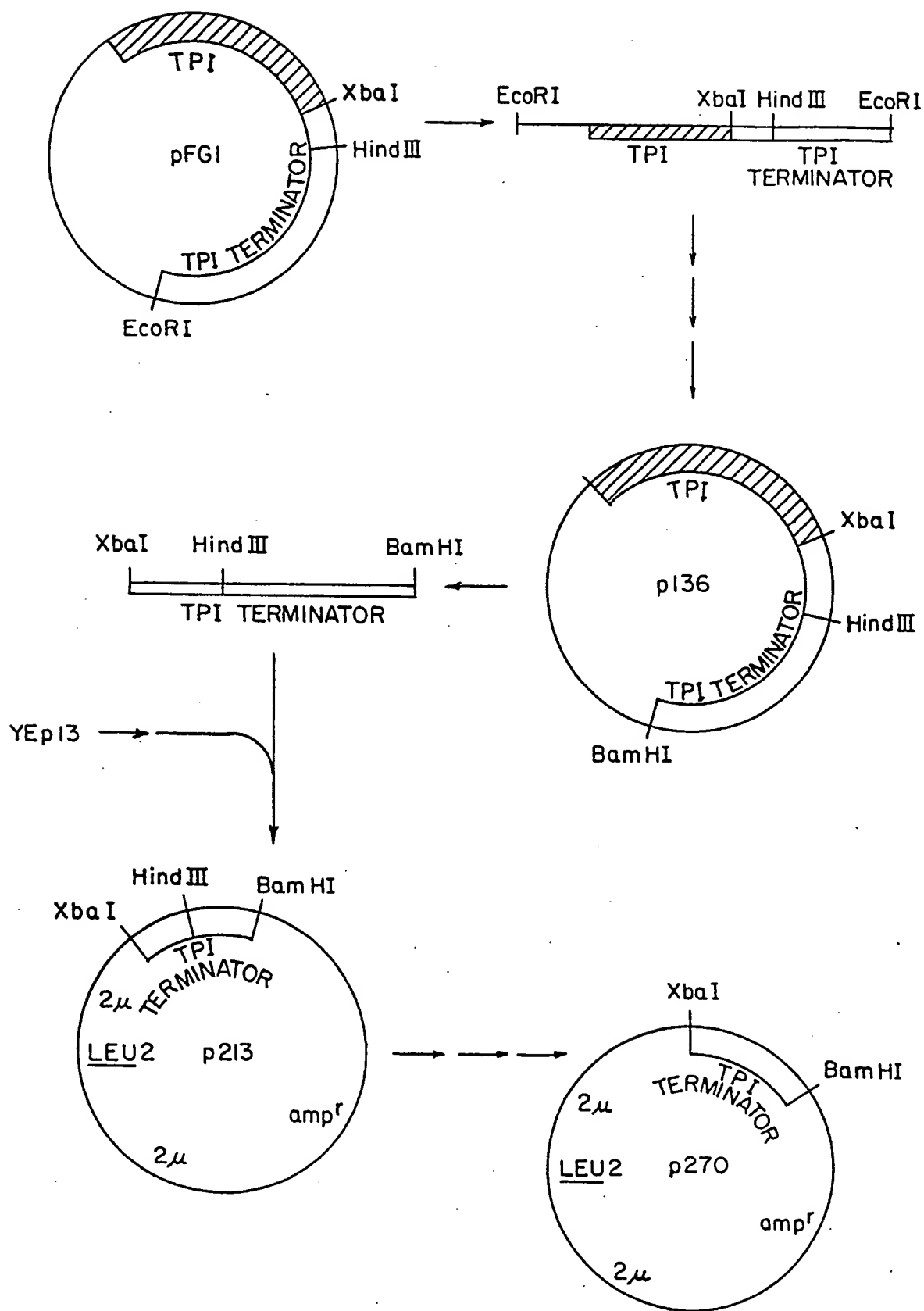
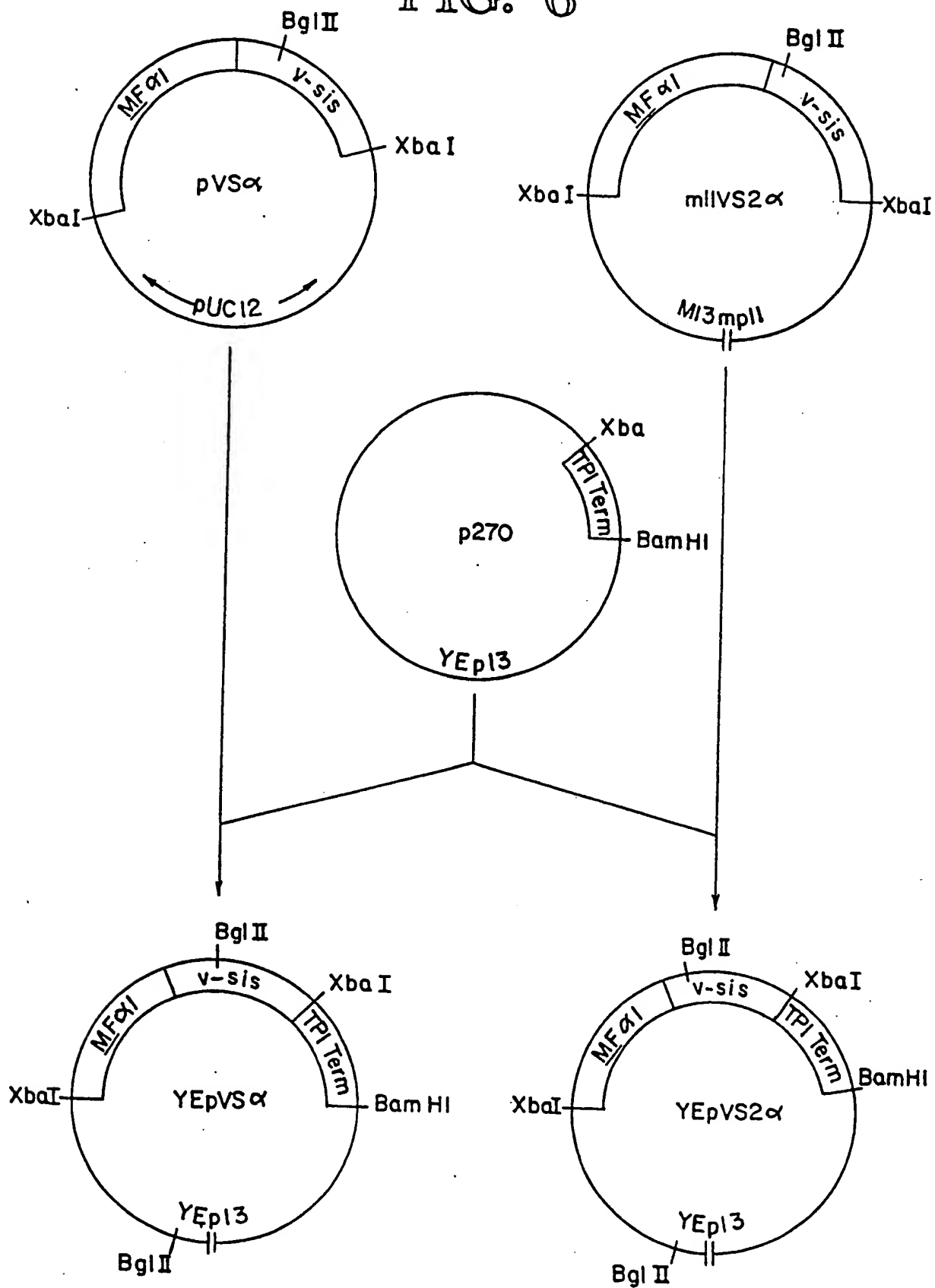


FIG. 6

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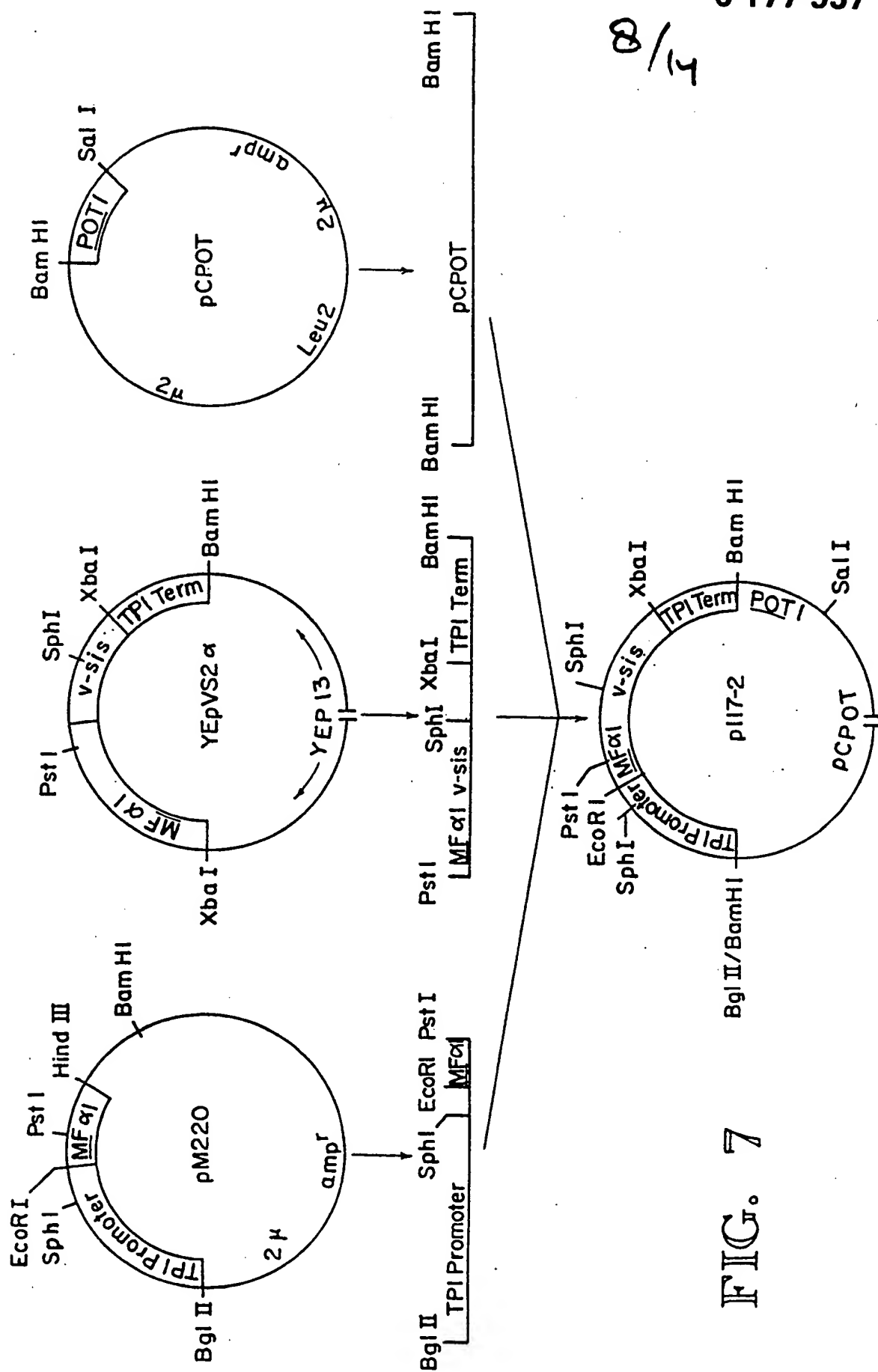
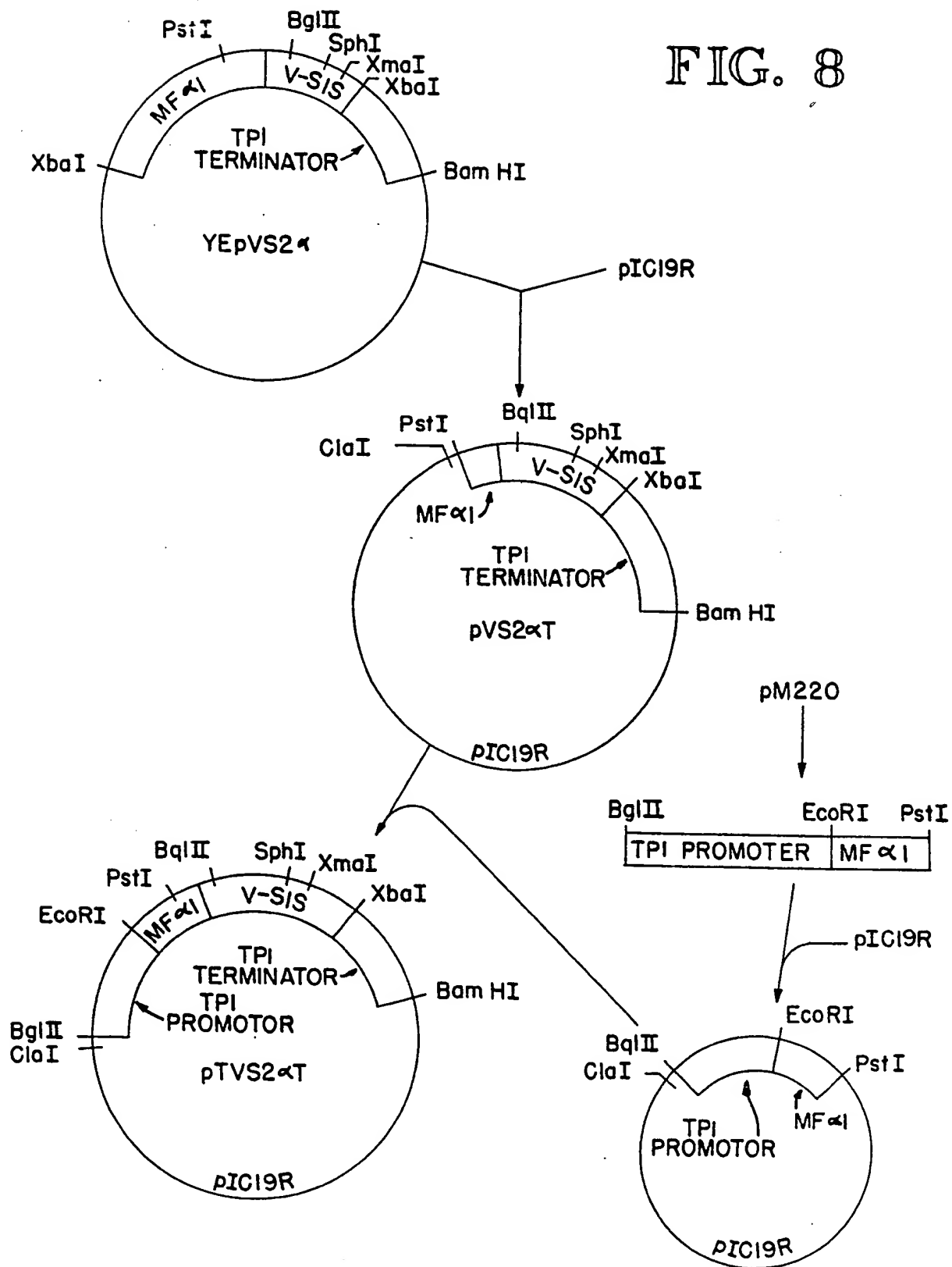


FIG. 7

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FIG. 8



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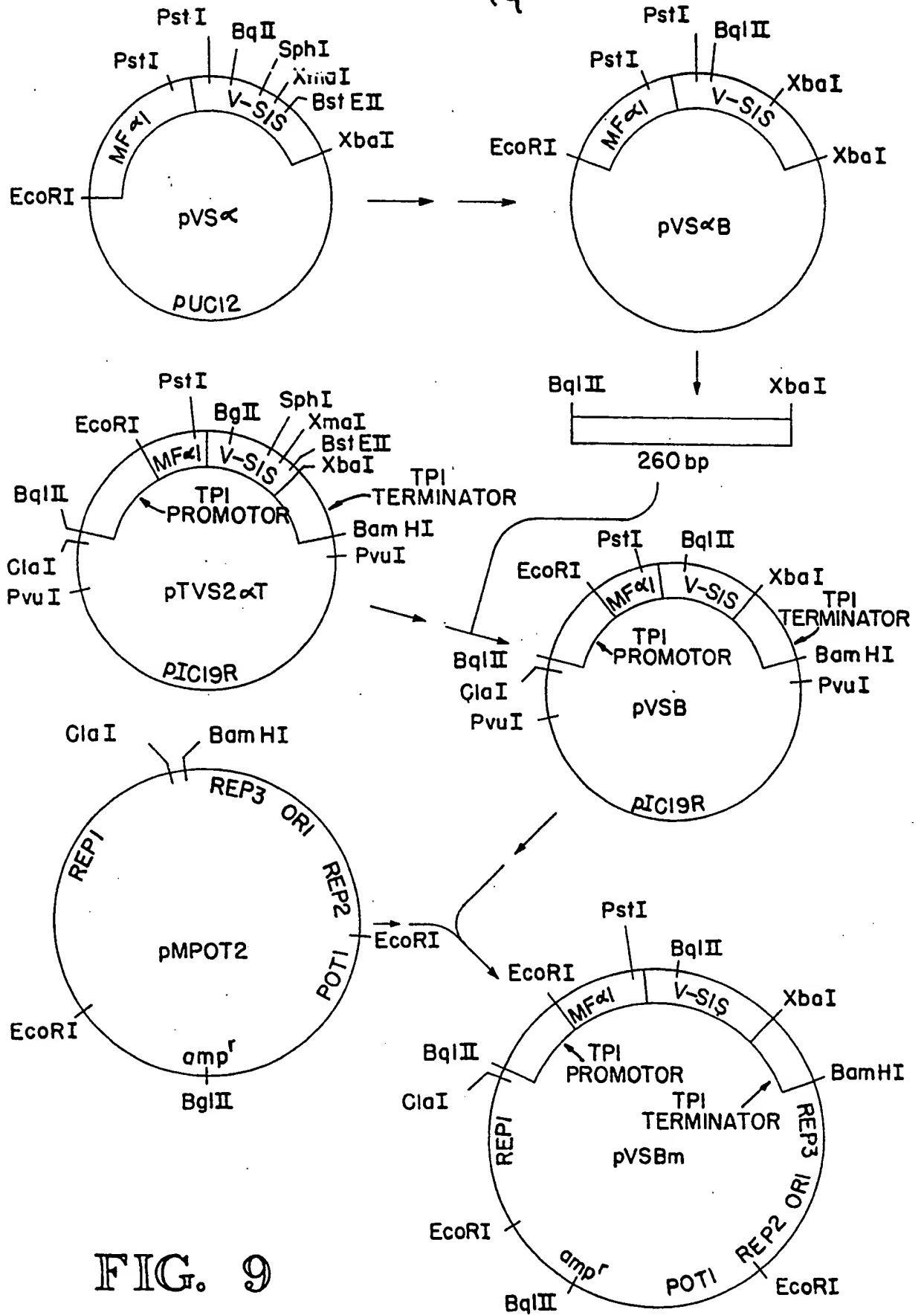


FIG. 9

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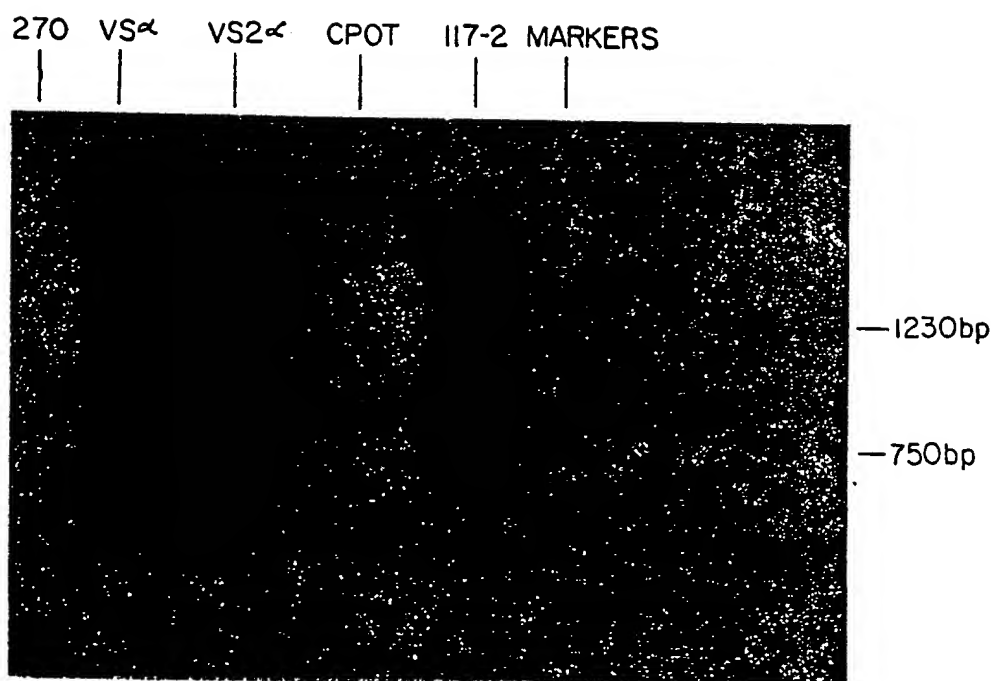


FIG. 10

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FIG. 11

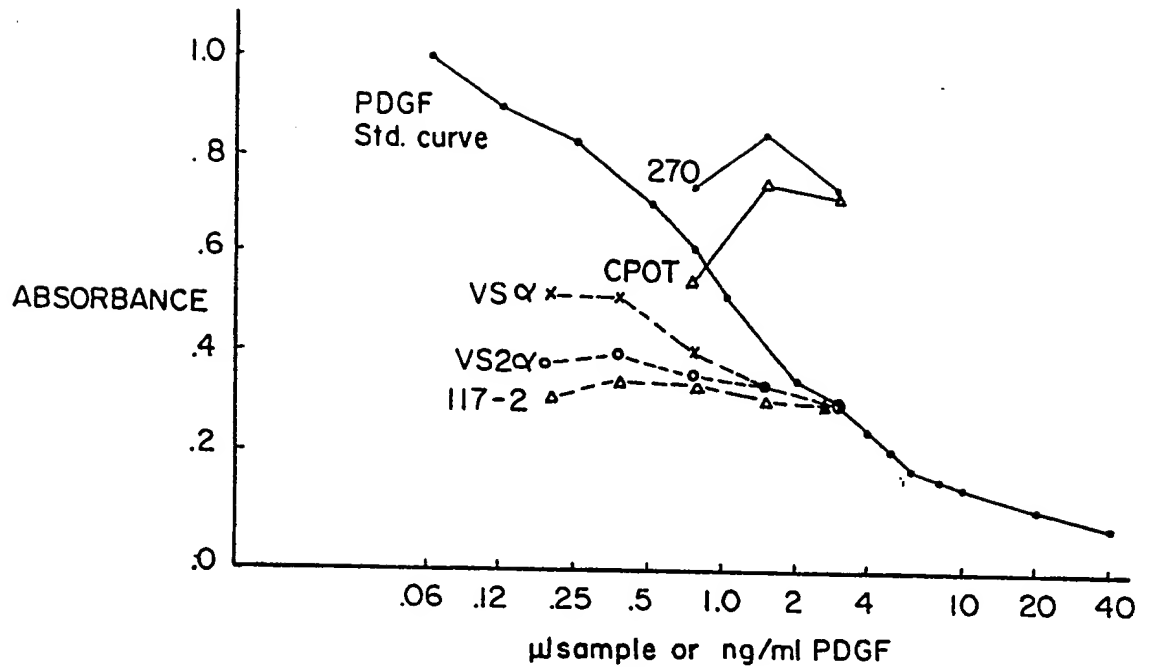
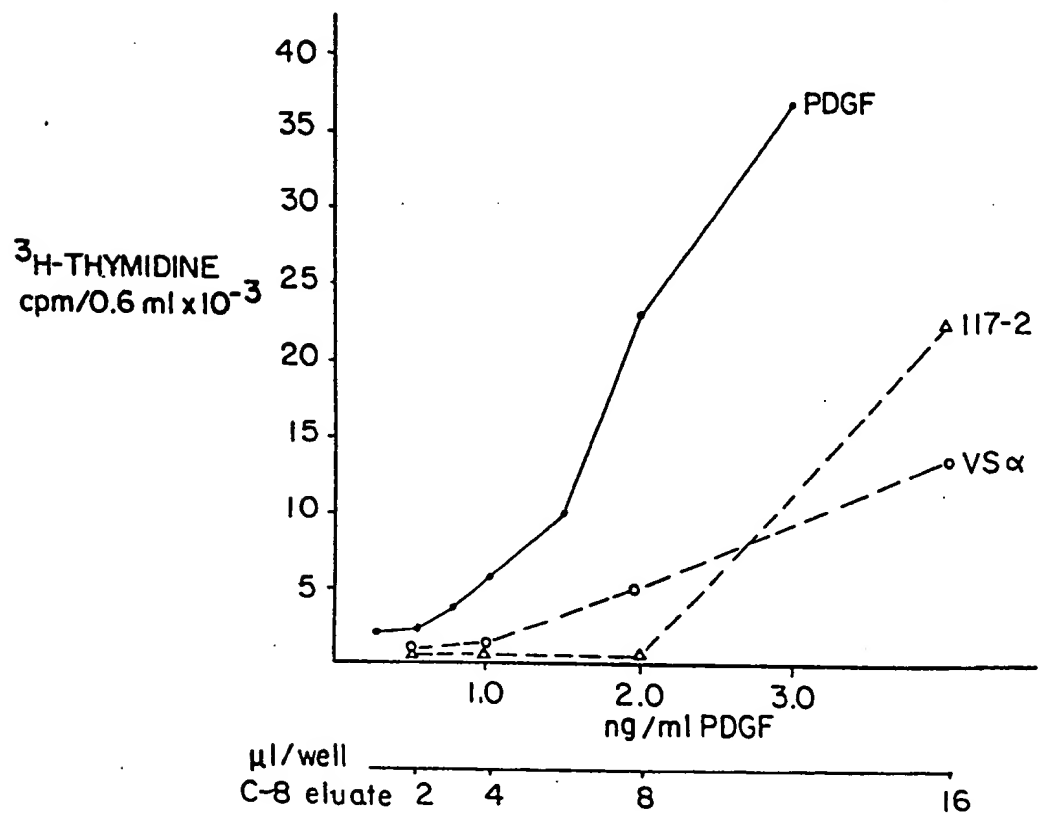


FIG. 12



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FIG. 13

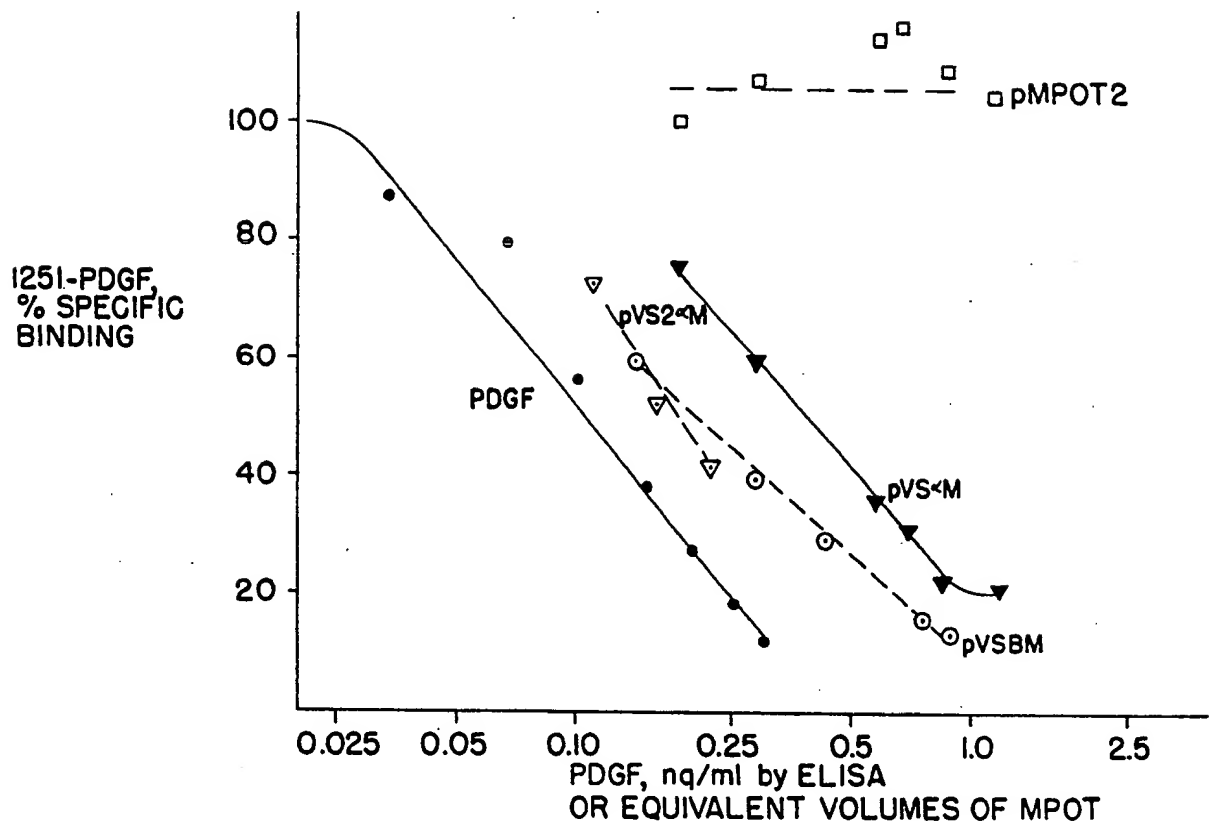
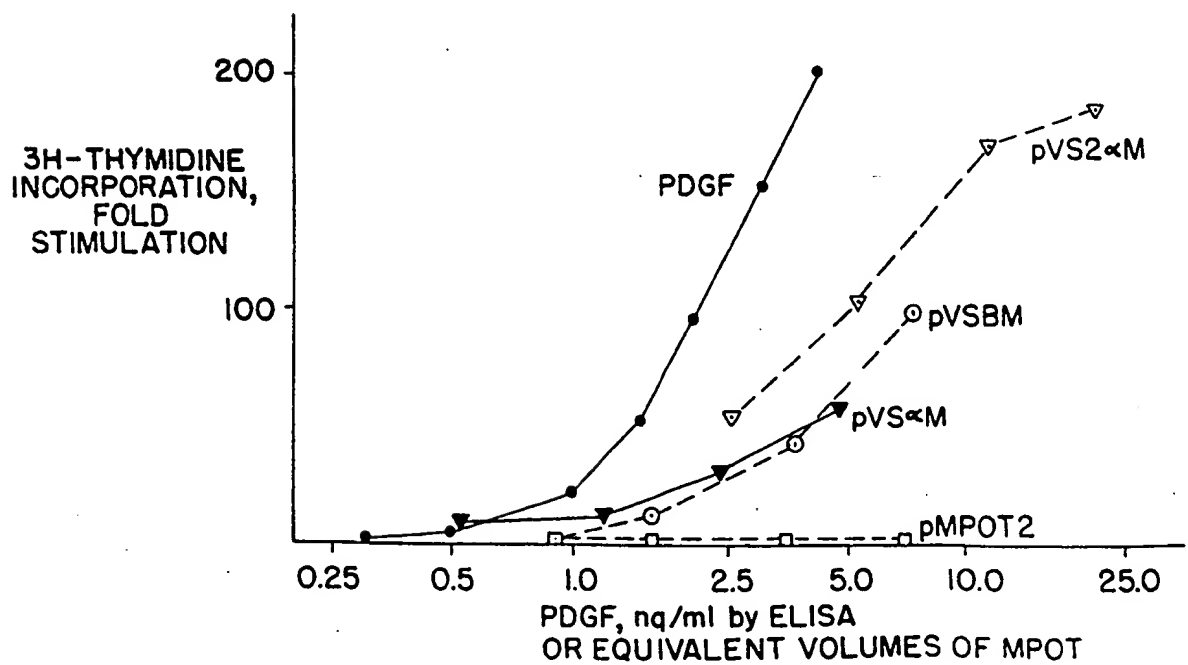


FIG. 14



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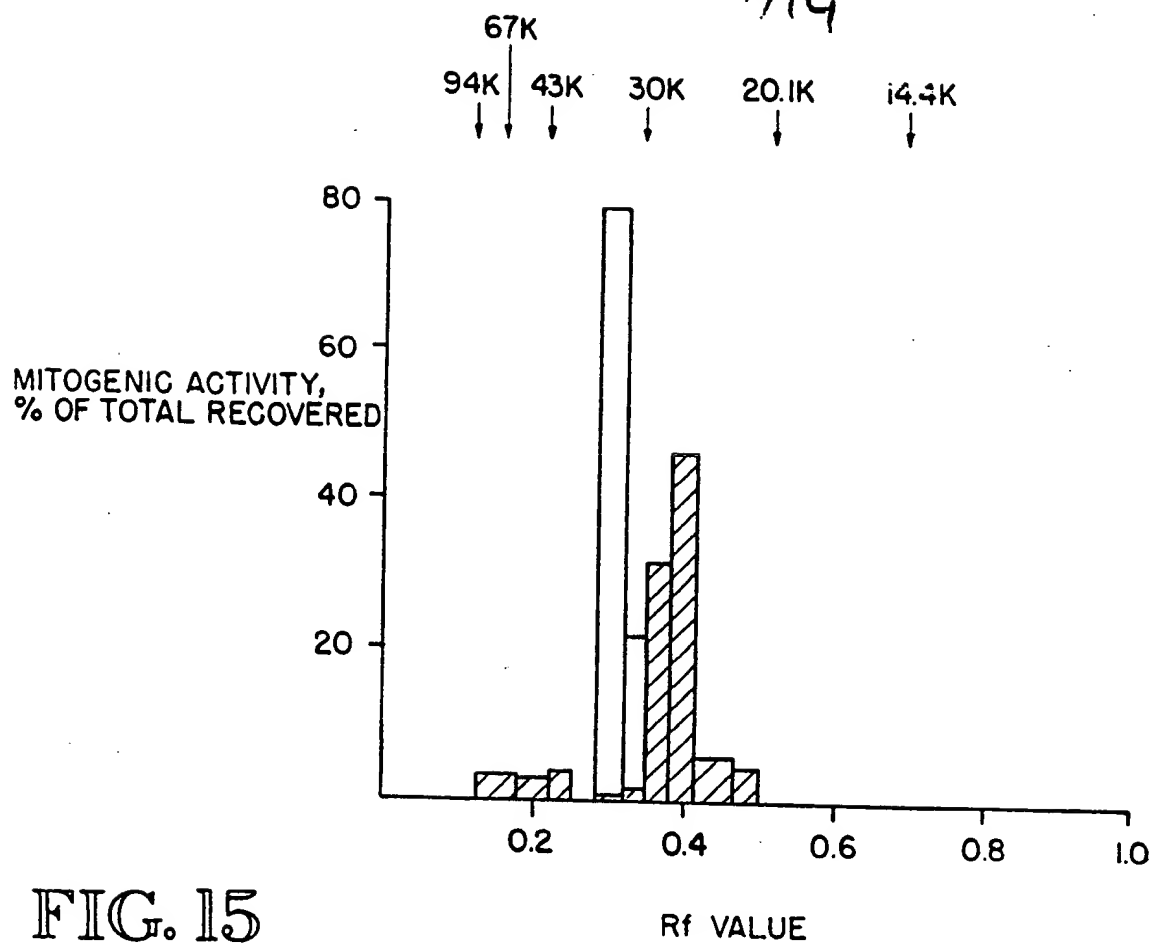


FIG. 15